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THE GASTRIC SECRETION OF BLOOD GROUP ANTIGEN IN RELATION TO ACID SECRETION.

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Received for publication June 19, 1952.

THE occurrence of blood group substances in body fluids has been known for a long time. Lehrs (1930) demonstrated the presence of A and B substances in the saliva of a proportion of individuals, and Schiff and Sasaki (1932) showed that the ability to secrete these substances in the saliva was controlled by a hereditary factor which behaves as a Mendelian dominant. The majority of individuals were designated " secretors " on the basis of the ABO system. Group O persons have not been shown, so far, to secrete an O substance, but may secrete another antigen to which the label " H " has been given by Morgan and Watkins (1948). Grubb and Morgan (1949) have shown that in the London population 76·89 per cent of individuals are secretors of A, B, or H.

Landsteiner and Levine (1926) first showed that A and B substances were present in tissue cells other than red cells and Schiff (1931) showed that these substances occurred in the tissues in two forms, water-soluble and alcohol-soluble. According to Friedenreich and Hartmann (1938) both forms are present in the tissues of " secretors," but in " non-secretors " the water-soluble form is either present in very small amount or is completely absent. Hartmann (1941) has shown that in " secretors " the antigen content is highest in glandular organs and that the stomach contains the largest quantity.

Considerable amounts of blood group antigen are secreted in the gastric juice of " secretors," equalling and sometimes exceeding the salivary content, but apart from the hereditary mechanism no reason for its presence has yet been found. Furthermore, nothing is known regarding which cells are responsible for the secretion of antigen or what changes might occur as a result of alteration of gastric function. The gastric mucous membrane contains four types of cells, oxyntic, peptic, mucus-secreting and argentaffin cells. The function of the latter is entirely unknown and there is no evidence that they contribute anything to the gastric juice. On the other hand, any of the other three types might conceivably be concerned with the secretion of antigen. In as much as blood group antigens

have been readily demonstrated in practically all tissues of the body, it may be supposed that all these cells will contain the antigen but not all will be capable of secreting it in its water-soluble form. The close chemical relationship of these antigens to mucin suggests that mucus-secreting cells are the likely source, and according to Maximow and Bloom (1948) these cells not only secrete a peculiar type of mucus, but show staining reactions somewhat different from, for example, those in the mouth. This, however, is pure supposition, and the origin of blood group antigen from the mucous glands cannot be accepted until other sources have been eliminated. The function of oxyntic and peptic cells is capable of control and assessment, and in the present investigation acid and antigen secretion were studied in order to determine whether there is any association suggesting a common origin.

MATERIAL AND METHODS.

The secretion of acid and antigen following an injection of histamine was studied in a group of 10 normal subjects. The procedure was as follows.

After an over-night fast the resting juice was drawn off through a Ryle's tube and saved. The stomach was then washed out with a measured volume of water. After all the washout fluid had been recovered a short interval was allowed to lapse, following which all further secretion was drawn off and saved. This constituted the pre-histamine specimen. An injection of 0·5 mg. histamine was then given subcutaneously. All secretion was drawn off at 5-min. intervals and measured. The first 5-min. sample was kept separate, but the 10- and 15-min. specimens were pooled, and likewise the 20- and 25-min. specimens and so on, giving final specimens for analysis which were labelled 5, 15, 25, 35, 45, and 55 min. respectively. By drawing off all secretion at 5-min. intervals loss from emptying of the stomach was avoided. Continuous salivary suction was maintained throughout to prevent swallowing of saliva.

A measure of the antigen content of the specimens was obtained by means of quantitative inhibition tests using natural anti-A and anti-B for group A and group B subjects and an immune anti-H prepared in rabbits for group O subjects. It was necessary to neutralize the juice before carrying out serological tests, not only because of haemolysis by acid, but on account of considerable differences in end-point over a wide range of pH on the acid side of neutrality. The addition of colour indicators was found to give variable serological results and had to be abandoned. Neutralization was carried out using the Cambridge pH meter, and as the volumes available were sometimes very small, it was found to be more convenient to use micro-electrodes for all titrations.

Most of the over-night resting juice specimens were found to be alkaline (pH 7·6–8·7). All acid specimens were adjusted to pH 7·0 by the addition of 0·2 N NaOH. The amount added was noted and the total vol. made up to a dilution of 1:2 with saline. The HCl content was calculated in m-equiv. per l. from the vol. of NaOH used. Calculation based on the pH related more to the state of affairs in stomach contents than to the actual secretion of acid. In so far as the titrations were carried to pH 7·0, the values given for HCl are not exactly comparable with the conventional ones for " free hydrochloric acid " using Töpfer's indicator. However, the difference is a slight one, and obviously makes no difference to the pattern of the response.

All specimens of gastric juice were found to be hypotonic in varying degree. Simple titrations of anti-A or anti-B in varying concentrations of sodium chloride showed no difference in end-point between 0·5 and 1·5 per cent. In the inhibition tests the progressive dilution with saline and the addition of serum ensured that hypotonicity did not interfere with the end-point.

In carrying out the quantitative inhibition tests an effort was made to standardize the antibody. The appropriate antibody (anti-A, anti-B or anti-H) was first titrated and the dilution giving the end-point (+) was arbitrarily regarded as containing one " unit." A dilution of antibody containing a standard number of units (16) was used in each test.

Before titrating the specimens for their antigen content they were centrifuged or filtered to remove débris. Parallel titrations were done on filtered and centrifuged specimens, and the results for two subjects indicated that, although the values for the centrifuged specimens

are persistently above those for the filtered, the difference was mainly one dilution only and the values ran parallel throughout. Filtering was henceforth carried out in all cases recorded.

The effect of hydrochloric acid on the antigen under the experimental conditions was studied. The saliva from a group A secretor was acidified (pH 1·03) and incubated for 4 hr. at 37°. Other specimens, after acidification, were allowed to stand at room temp. and in the cold for several days. After neutralization the specimens were titrated in parallel with the untreated saliva. The results of such an experiment indicate that there is no difference in titre after 4 hr. at 37° and a barely significant fall after 5 days at room temp.

In separate experiments saliva was collected from a number of subjects at intervals following an injection of histamine. The specimens were immediately placed in a boiling water bath for about 5 min., filtered and immediately titrated.

The end point has been taken as the highest dilution of gastric juice or saliva causing complete inhibition of 16 " units " of antibody.

RESULTS.

A typical result obtained in the titration of antigen in one subject is given in Table I.

TABLE I.—*Titration of Antigen in Gastric Juice following Histamine* (*Subject* 1).

Specimen.	Dilutions of gastric juice (powers of 2).											Saliva controls.	
	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.		
Resting juice .	−	−	−	w	+	+	+	+	++	+++	+++	.	−
Before histamine	−	−	−	−	w	w	+	+	+++	+++	.	.	−
5 min. .	−	−	w	w	+	+	++	++	+++	.	.	.	−
15 ,, .	−	−	−	−	w	w	+	+++	+++	.	.	.	−
25 ,, .	−	−	−	−	w	w	+	++	+++	.	.	.	−
35 ,, .	−	−	w	+	+	+	+	+++	+++	.	.	.	−
45 ,, .	−	−	−	w	+	+	++	++	+++	.	.	.	−
55 ,, .	−	−	−	−	w	+	+	++	+++	.	.	.	−

+++, ++, +, w, degrees of agglutination ; −, no agglutination.

Dilutions have been expressed in terms of logarithms to the base 2 and on this basis Table II has been constructed summarizing the results in the whole group, together with the corresponding acid concentrations and the volumes of the specimens.

It will be seen that while antigen titres have varied in some cases to a serologically significant degree (in subject 4 the range of variation was 4 tubes), there is no constant pattern in relation to the acid concentrations.

It was desirable to determine whether histamine had any direct influence on the secretion of antigen, and the effect on salivary secretion was clearly relevant evidence on this point. Table III summarizes the results obtained in 10 subjects. The antigen secretion in the saliva was not significantly altered by histamine.

DISCUSSION.

It is clear from the above results that histamine does not directly stimulate antigen secretion. The salivary secretion of antigen did not appear to be affected by the administration of histamine, and had there been any such effect on gastric secretion of antigen there should have been a rise in antigen concentration in parallel with the acid curve. No such rise was observed.

TABLE II.—*Comparison of Antigen and Acid Secretion in Gastric Juice of* 10 *Subjects.*

Subject.				Time of taking specimens.							
				Overnight resting.	Before histamine.	Min. after histamine.					
No.	Sex.	Group.				5.	15.	25.	35.	45.	55.
1.	F.	A	HCl	0	2·1	0	18·9	27·9	10·5	12·6	2·1
			Antigen	6	7	5	7	7	5	6	7
			Volume.	20	5	6	20	13	22	17	13
2.	F.	O	Ditto	0	0	35·3	88	103	101	80	3·14
				4	3	4	3	3	4	3	3
				20	5	6	18	24	25	19	30
3.	F.	A	„	0	2·1	21	56·5	49·3	60	65	39
				2	—	4	5	4	3	2	4
				20	2·5	17	22·5	25	22·5	37	15
4.	F.	B	„	0	0	0	17·8	50·5	60	59	48·5
				10	12	11	10	10	9	9	8
				33	33	15	30	30	23	25	10
5.	F.	A	„	1·05	32·5	56	77	92	87	92	72·5
				5	5	6	6	5	5	5	—
				50	7	5	21·5	38·5	22	21·5	10
6.	F.	O	„	1·05	35·7	34·5	78	101	109	107	—
				4	5	5	5	3	4	4	—
				35	16	11	28	27·5	26	21·5	—
7.	M.	O	„	0	0	0	0	0	0	0	—
				7	7	7	7	5	6	7	—
				28	5·5	6·5	10	12	12	16	—
8.	M.	A	„	2·1	75	82	106	109	116	109	92
				3	4	3	3	3	2	2	—
				24	15	17·5	30	47·5	37	27	14
9.	M.	A	„	0	12·6	25·2	80	87	91	—	—
				6	7	7	7	6	5	—	—
				26	6	13·5	50	21	25	—	—
10.	M.	A	„	77	113	115·5	122·5	114·5	98·5	45	—
				7	6	6	5	5	6	8	—
				115	20	24	62	51·5	47·5	15·5	—

HCl expressed in m-equiv. per l.

Antigen expressed in terms of highest dilutions of juice inhibiting completely 16 " units " of antibody, *e.g.*, 6 = 1:2^6 or 1:64.

Volume measured in ml.

TABLE III.—*Concentration of Blood Group Antigen in Saliva after an Injection of Histamine.*

Subject.			Time of taking specimens.					
			Before histamine.	Min. after histamine.				
No.	Sex.	Group.		5.	15.	25.	35.	45.
9	M.	A	8	7	6	6	8	7
11	F.	A	8	8	9	7	8	8
12	F.	A	7	7	7	6	6	6
13	M.	O	6	6	6	6	7	6
14	M.	O	6	5	5	5	4	5
15	M.	A	3	4	3	3	4	3
16	M.	O	5	6	5	4	6	5
17	M.	AB*	2	1	1	1	0	1
18	M.	A	6	6	4	5	4	5
19	M.	O	6	4	4	3	5	5

Antigen content expressed as in Table II.

* Titration of A antigen.

If the rate of secretion of antigen remained constant throughout the experiment, then the flow of acid juice under the influence of histamine should have resulted in a fall in antigen concentration due to dilution. A significant fall rarely occurred. In Table II a study of the antigen concentrations and volumes of juice show that although 2- and 3-fold differences in volume have occurred, corresponding antigen concentrations have been variable and in any case barely within the range of significance. In fact, a rise in antigen concentration is sometimes associated with an increase in volume, and a fall with a decrease, as in subjects 4 and 9. This indicates that an increase in antigen secretion has occurred. This increase has not been provoked directly by histamine, but might be due to the presence of acid. Comparison of antigen and acid concentrations, however, show that they are quite unrelated, and the independence of acid and antigen secretions is illustrated even more definitely in subject 7, who showed a histamine-fast achlorhydria with a steady secretion of antigen throughout the test. The conclusion to be drawn from these experiments is that the secretion of blood-group antigen is not related to the activity of the oxyntic cells. Attention is therefore turned to the peptic cells.

It is believed by many that histamine has no direct influence on the activity of the peptic cells, and that the short-lived rise in pepsin content of gastric secretion following an injection of histamine is due to the washing of pepsin out of the glandular ducts. In order therefore to investigate the relationship of antigen secretion to peptic activity, an accepted stimulant of pepsin secretion was utilized.

Insulin has long been known to produce a flow of juice rich in pepsin, and insulin "test-meals" are being carried out on normal subjects. The results so far obtained in a small number of cases suggest that there is no correlation between antigen and pepsin secretion.

SUMMARY.

The gastric secretion of acid and blood group antigen was investigated by means of histamine "test-meals." Histamine had no demonstrable effect on the secretion of antigen, which appeared to be independent of the secretion of acid.

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PROTEIN AND NUCLEIC ACID CONTENTS OF RAT LIVERS ONE DAY AFTER A SINGLE INJECTION OF CARBON TETRACHLORIDE.

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In normal rats the amount of deoxyribonucleic acid (DNA) present in the liver is determined by the body-weight and is not affected by variations in the protein, fat and choline contents of the diet (Campbell and Kosterlitz, 1950, 1952*a*; Mandel, Jacob and Mandel, 1950) or by fasting up to 48 hours (Davidson, 1947). Further, the DNA content of liver nuclei of adult rats is not altered by prolonged fasting (Mirsky and Kurnick, 1951), or by feeding of a protein-free diet for periods up to 4 weeks (Campbell and Kosterlitz, 1952*b*). This constancy of liver DNA makes it a very suitable standard of reference for other liver constituents. Thus, the ribonucleic acid (RNA) content of an arbitrary but constant number of liver cells can be stated by referring RNA P to 1 mg. DNA P. It is then found that the RNA content of the liver cells varies linearly with their protein content (protein N/DNA P), and that, by means of a regression equation, the RNA content can be predicted for any given protein content (Campbell and Kosterlitz, 1950, 1952*a*).

In view of these findings it seems worth while to examine how far the DNA, RNA and protein contents of the liver are affected by carbon tetrachloride. Campbell and Kosterlitz (1948) have found that as early as 24 hours after injection of carbon tetrachloride there is a rise in liver weight which is due to an increase of the protein, phospholipin and nucleic acid contents. One of the earliest changes seen in the liver after injection of carbon tetrachloride is the decrease of cytoplasmic basophilia in the central parts of the lobules (Cameron and Karunaratne, 1936; Rosin and Doljanski, 1946). This finding suggests a loss of RNA which is the cytochemical basis of the basophilia. Chemical analysis, however, shows that liver RNA is increased (Farber, Koch-Weser, Szanto and Popper, 1951).

METHODS.

Experimental procedure.

Male hooded rats, 3 to 4 months old, of the Rowett Institute strain were used. The basal diet consisted of 2 per cent agar, 3 per cent salts, 10 per cent cooking fat and 85 per cent sucrose. Where casein (vitamin-free, Glaxo) was given, it replaced an equal weight of sucrose. The fat was supplemented with vitamins A, D and E (Kosterlitz, 1947). If not stated otherwise, 6·3 g. food/100 g. body weight was offered, corresponding to 25 kcal. Immediately before feeding time at 4 p.m. each rat was injected subcutaneously with a solution in 0·5 ml. of the following quantities of water-soluble vitamins: 5 μg. biotin, 5 μg. folic acid, 20 μg. synkavit dicalcium phosphate (Roche), 30 μg. aneurin, 30 μg. pyridoxin, 50 μg. riboflavin, 100 μg. calcium D-pantothenate, 100 μg. nicotinic acid, 100 μg. *p*-amino-

benzoic acid, 1 mg. inositol and 17·3 mg. choline hydrochloride. In the experiments in which rats were fed on sugar and 10 per cent fat for 2 days, no vitamins were added. Carbon tetrachloride (B.D.H., Analar) was given as a single subcutaneous injection of 0·2 ml./100 g. initial body weight 24 hr. before the rats were killed. It would have been of interest also to examine livers 48 and 72 hr. after carbon tetrachloride, but the infiltration of round cells rich in DNA would have made interpretation difficult.

Analytical methods.

These have already been described (Kosterlitz, 1947; Campbell and Kosterlitz, 1948, 1949).

Calculation of results.

The DNA P values are given in mg. per liver. The observed values are compared with those predicted from the equation DNA P (mg./liver) = 0·00518 × body-weight (g.) + 0·755 (Campbell and Kosterlitz, 1950). All values are adjusted to a body-weight of 340 g., for which DNA P is calculated to be 2·52 mg./liver.

The values of protein N and RNA P are referred to 1 mg. DNA P. In normal rats, but not necessarily in rats injected with carbon tetrachloride, this represents a constant number of liver nuclei. The observed values of RNA P/DNA P are compared with those predicted from the equation RNA P/DNA P (mg./1 mg.) = 0·02763 × protein N/DNA P (mg./1 mg.) + 1·21. All values are adjusted to a protein N/DNA P of 100 mg., for which RNA P/DNA P is calculated to be 3·97 mg.

The significance of the difference between observed and predicted values is calculated as previously described (Campbell and Kosterlitz, 1950).

Histological technique.

Liver tissue was fixed in 5 per cent neutral formaldehyde in 0·9 per cent NaCl. Ester wax sections were stained with haemalum without counterstain. This made possible an estimation of cytoplasmic basophilia.

RESULTS.

In rats fed on adequate amounts (22–24 kcal.) of the protein-free or 23 per cent casein diets, injection of carbon tetrachloride caused within 24 hr. a rise in the DNA and RNA contents of the liver (Table). While the increase in liver DNA was small, ranging between 6 and 7 per cent of the predicted values, the rise in liver RNA was very considerable, amounting to almost 20 per cent.

Fasting of control rats for 48 hr. did not affect liver DNA but the RNA content of the liver cells was lowered. Injection of carbon tetrachloride in such rats caused increases in both DNA and RNA, the rise of the latter being, however, smaller than in non-fasting rats. When a mixture of sugar and fat, without vitamins, was fed instead of fasting the rats, the DNA and RNA values of the control rats were normal. In this group of animals carbon tetrachloride caused no rise in DNA, but an increase in RNA of the order found in rats fed on adequate diets. The protein content of the liver cells was raised a little in the rats fed with the sugar-fat mixture but not in the fasted rats.

In order to examine the effects of reduced food intake on the liver changes caused by carbon tetrachloride, rats were fed on diets containing 0, 23 and 70 per cent casein in amounts corresponding to 13 kcal./100 g. body weight. The DNA and RNA contents of the control animals with reduced food intake were lower than the values predicted from the regression equations; carbon tetrachloride raised both DNA and RNA values to about the same extent as in rats with unrestricted food intake. There was no rise of the protein content of the liver cells.

TABLE.—*Liver Protein and Nucleic Acids 24 hr. after Injection of Carbon Tetrachloride.*

Diet	Control or CCl_4.	Body weight		Daily food intake before/after CCl_4 (kcal./100 g. body weight).	DNA P.			Protein N/DNA P (mg./1 mg.).	RNA P/DNA P.		
		Initial.	Final. (g.).		Observed.	Adjusted to body-weight of 340 g. (predicted DNA P = 2·52) (mg./liver).	Change after CCl_4.		Observed.	Adjusted to protein N/DNA P of 100 (predicted RNA P = 3·97) (mg./1 mg.).	Change after CCl_4.
Protein-free, 4 days	CCl_4	338	310	22/10	2·70	2·71	+0·19**	98	4·71	4·76	+0·79***
23 per cent casein, 4 days	CCl_4	301	320	22/21	2·58	2·68	+0·16*	132	5·64	4·76	+0·79***
23 per cent casein, 2 days followed by 48-hr. fast	Control	329	289	23	2·35	2·41NS		99 NS	3·62	3·65***	
	CCl_4	331	293	24/0	2·78	2·83	+0·42***	102	3·93	3·87	+0·22*
23 per cent casein, 2 days sugar and 10 per cent fat, 2 days	Control	328	312	22	2·57	2·63NS		106 *	4·03	3·86NS	
	CCl_4	336	315	24/16	2·54	2·56	−0·07NS	118	4·97	4·47	+0·61***
Protein-free, restricted, 10 days	Control	343	284	13	2·37	2·35**		79 NS	3·28	3·86NS	
	CCl_4	339	283	13/10	2·62	2·63	+0·28**	84	4·26	4·70	+0·84***
23 per cent casein, restricted, 10 days	Control	345	289	13	2·35	2·32***		100 NS	3·69	3·69***	
	CCl_4	345	302	13/11	2·62	2·59	+0·27***	103	4·28	4·20	+0·51***
70 per cent casein, restricted, 10 days	Control	338	279	13	2·36	2·37*		108 NS	3·66	3·44***	
	CCl_4	340	283	13/13	2·56	2·56	+0·19*	108	4·32	4·10	+0·66***

Each value is the mean obtained from 4 rats, or 5 rats in the case of the 23 per cent casein diet, low food intake. The DNA P values have been adjusted to a body-weight of 340 g. by means of the regression coefficient 0·0518, and the RNA P/DNA P values to a protein N/DNA P of 100 by means of the regression coefficient, 0·02763 (Campbell and Kosterlitz, 1950). Significance of differences from predicted values or of change after carbon tetrachloride: NS = not significant; *, **, *** = $P < 0·05$, 0·01, 0·001 respectively.

Histological changes.

In the animals eating adequate amounts of food the changes were similar to those observed previously (Campbell and Kosterlitz, 1948). Large balloon-like hydropic cells were present in 1 out of 4 rats fed on the protein-free diet and in 2 out of 3 rats fed on the 23 per cent casein diet. When the food intake was reduced no hydropic cells were found in the livers of the rats fed on the protein-free diet, while they were present in the livers of 4 out of 5 rats fed on the 23 per cent casein diet and 3 out of 4 rats fed on the 70 per cent casein diet. There were hydropic cells in the livers of 3 out of 4 rats fasted for 48 hr., but only in 1 out of 4 rats fed on sugar and fat for the last 48 hr.

The livers of all rats showed a decrease of cytoplasmic basophilia in the central and an increase in the peripheral areas of the liver lobules. This was also found in livers with little sign of injury and sometimes appeared to be the only pathological change. Although in the fasted rats the increase in chemically determined RNA was smaller than in fed animals, the cytoplasmic basophilia of the periportal area did not seem to be less pronounced than in the other groups of rats.

In many of the livers round-cell infiltration was present, but a correlation between the number of round cells and the rise in liver DNA could not be established with certainty. For instance, in the rats fasted for 48 hr. the increase in liver DNA seemed to be associated with a considerable number of round cells, and in the rats fed on sugar and fat for 48 hr. there was no increase in either round cells or DNA. On the other hand, in the animals with reduced food intake the round cells were very numerous in the rats fed on the protein-free diet and much less and sometimes absent in the other two groups. The increase in liver DNA did not differ significantly between these groups.

DISCUSSION.

In a previous paper the authors (Campbell and Kosterlitz, 1948) showed that the rise in liver weight found as early as 24 hours after injection of carbon tetrachloride was due to an increase in the protein, phospholipin, nucleic acid and water contents of the liver. The suggestion was then made that these changes taken together with the increased incidence of mitoses pointed to a formation of new liver cells.

This view is supported by the rise of liver DNA. There are, however, other possible interpretations. First, there may be a rise in the DNA content of the average liver cell, either by an increase in polyploidy or in the number of binucleated cells. Neither possibility has been followed up so far. Secondly, there is the infiltration of round cells. The histological evidence is equivocal, since there is no definite correlation between the increase in liver DNA and number of round cells. Estimations on leucocytes from human blood show that their RNA content is approximately one-tenth of that of rat or human liver cells (Davidson, Leslie and White, 1951). Therefore livers with large numbers of round cells should have lower ratios of RNA P/DNA P than livers with no significant infiltration, which is not the case. Thus the increase in liver DNA cannot be solely brought about by round-cell infiltration.

The observation of Farber *et al.* (1951) that the mean RNA content of the liver cells is markedly increased after carbon tetrachloride is confirmed. The protein and energy contents of the diet have no influence on the magnitude of this increase. Fasting for 48 hours, however, causes this rise to be smaller than in the rats fed

on normal or half rations. Farber *et al.* pointed out the difficulty in reconciling the increase in liver RNA with the loss in cytoplasmic basophilia in the central areas of the lobules, and drew attention to the possibility that the cells in the periphery may contain increased amounts of RNA. The evidence presented here supports this conception. The cytoplasm of the peripheral cells appears to stain more intensely with haemalum in rats injected with carbon tetrachloride than in control rats fed on the same diet. It may be added that the reduction in cytoplasmic basophilia in the cells of the central areas does not necessarily mean that these cells contain little or no RNA, which may no longer be demonstrable by histological methods. Finally, the increased number of round cells cannot contribute to the rise in RNA, since the RNA content of leucocytes is very much smaller than that of liver cells (Davidson *et al.*, 1951).

The results of chemical analysis of liver tissue presented in this and the earlier paper have thus confirmed and extended the findings obtained by histological methods. Some of the rise in liver weight found 24 hours after carbon tetrachloride is undoubtedly caused by the increased triglyceride and water contents, but most of it is due to the formation of new liver cells, as shown by the increased number of mitoses and the increases in liver DNA, protein, phospholipin and RNA. The protein and phospholipin contents of the new liver cells are similar to those of the original cells, *i.e.*, they are higher on diets containing adequate amounts of protein than on protein-free diets. The significance of the high RNA contents of the new liver cells is not quite clear, but may possibly be associated with an increased intensity of protein synthesis. Such a relationship between protein synthesis and RNA was first suggested by Caspersson (1941). In this connection it may be of significance that a similar increase in RNA with a similar localisation is found in pregnant rats' livers. In this instance a factor secreted by the placenta is the responsible agent (Campbell and Kosterlitz, 1949).

SUMMARY.

An increase in liver deoxyribonucleic acid and a rise in the ribonucleic acid (RNA) content of the liver cells of male rats is found 24 hr. after a single subcutaneous injection of carbon tetrachloride. Total liver protein rises, but the protein content of the individual liver cells remains unchanged.

These changes are independent of the protein and energy contents of the diet, and are interpreted as indicating an increase in the number of liver cells due to regenerative processes in the periphery of the liver lobule. The protein and phospholipin contents of the new cells are similar to those of the original cells and are determined by the amount of protein eaten. It is likely that the increase in the mean RNA content of the liver cells is due to a high RNA content of the cells of the periphery of the liver lobule.

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TRYPAN BLUE-INDUCED TUMOURS OF RATS.

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GILLMAN, Gillman and Gilbert (1949) reported reticulum cell sarcomas and other lesions in the livers of rats stained for a long period with Trypan blue. The occurrence of lymph-node tumours was also noted (Gillman and Gillman, 1949).

The present paper reports a similar experiment using a different brand of the dye. The same proportion, over 50 per cent, of the rats developed liver tumours. The lymph-node tumours and some of the other liver lesions were not observed.

MATERIAL AND METHODS.

The rats were bred in the laboratory from a May & Baker hooded strain. At the start of the experiment they were about 3 months old and weighed 90–150 g. Their food was a mixture of rat chow, bread, oats and greens. There were 53 males and 48 females, 24 of each being kept as controls. One ml. of a 1 per cent solution of Trypan blue B.P.C. (Avlon brand, I.C.I.) dissolved in cold sterile distilled water was injected subcutaneously in the groin every 2 weeks. In 37 animals the dye was continued until death. In 16 the injections were discontinued after a total dose of 140–160 mg. All 16 had laparotomies and liver biopsies before the dye was stopped.

The rats were allowed to die naturally or were killed when death seemed imminent. At post-mortem pieces of liver were removed for histological examination in all cases and an extensive survey was made of the other organs, including seven groups of lymph nodes.

TABLE.—*Tumour Incidence in Rats Treated with Trypan Blue.*

Rats receiving 10 mg. Trypan blue fortnightly until death.			Rats receiving 14 injections of 10 mg. Trypan blue over 182 days.		
Survival in days.	With tumour.	Without tumour.	Survival in days.	With tumour.	Without tumour.
151–250	12	9	210–250	3	2
251–350	6	3	251–300	3*	1*
351–450	0	1	301–350	0	2*
451–550	1	0	351–400	1	3†
			401–450	0	1
Total	19	13		7	9

* One rat received 16 injections of 10 mg. over 210 days.
† Two rats received 16 injections of 10 mg. over 210 days.

RESULTS.

Length of survival and tumour incidence are stated in the Table. Five stained and 2 control rats died before the 150th day and were free of tumours. Twenty-six of the 48 experimental rats which survived this period developed reticulum cell sarcoma of the liver. The first tumour caused death 153 days after starting the dye ; the average period for the 14 males was 221 days, and for the 12 females 268 days. No tumours occurred among the control rats.

Gross findings.

Infections were frequent, but showed no difference in incidence between stained rats and controls. Ascites occurred in all the tumour-bearing rats except two, in which only solitary tumours were found. Small pleural effusions and a widespread tissue oedema were often present with the ascites. The liver and spleen were usually a little enlarged in the stained group and, where tumours were present in the liver, the spleens were about twice the size of those in the other stained rats. The tumours presented as multiple nodules throughout the liver, varying in diameter from 1–25 mm. They appeared a brighter blue against the blue-brown back-ground of liver tissue. Lymph nodes were a little enlarged in the stained rats and, in two of those with tumours, lymph nodes along the upper border of the pancreas appeared to contain metastases. Some degree of atrophy of the testes was common in the stained animals. The other organs showed no abnormal features.

Histology.

In the early weeks of staining there was an apparent increase in the numbers of Kupffer cells in the liver. This was associated with some histiocytic proliferation in the portal tracts, though these cells did not store as much dye as the Kupffer cells. In all the rats which survived 100 days of staining this proliferation became so marked, especially around the origins of the smallest bile ducts and vessels, that all but the largest portal tracts appeared scalloped on section (Fig. 1). Small cysts containing free histiocytes and a little proteinous fluid were sometimes seen and appeared to be lined by Kupffer cells (Fig. 2). Necrosis of individual liver cells in the limiting lamina around the portal tracts was occasionally present. Though there was no apparent communication with the blood stream, a few erythrocytes appeared in the cysts and were phagocytosed.

Sometimes this picture persisted unaltered in parts of the livers of animals with multiple tumours. In others the neat scalloped formation around the portal tracts was lost, the infiltration became more diffuse, and the small cysts collapsed or were overgrown by proliferating cells. At the same time histiocytes frequently collected around the hepatic veins. In one rat with a solitary tumour, compact small groups of abnormal cells were found among the proliferating histiocytes and were strongly suggestive of multiple foci of tumour origin. There was no definite time relationship in these changes, all types of picture being seen from about the 150th to the 450th day.

The tumour nodules consisted of polygonal cells of varying size. These bore a striking resemblance to Kupffer cells, though the nucleus was usually larger and frequently elongated to form a horseshoe or more bizarre shape (Fig. 3). In places the tumour was highly vascular and it was not always possible to distinguish a separate vascular endothelium. Large quantities of reticulin were usually formed,

sometimes outlining groups of cells, but not infrequently around individual cells (Fig. 4). Cyst formation was frequent, and the free cells resembled the sinus phagocytes of lymphatic tissue. Around the tumour nodules the columns of liver cells were frequently separated by two or three layers of tumour cells lying both within and without the sinusoidal reticulin. Necrosis of individual liver cells and occasionally of whole columns followed. The tumour cells were intensely phagocytic and contained fragments of liver cells, Trypan blue, erythrocytes, and large quantities of fat. Mitosis was generally infrequent. Single surviving liver cells and small bile ducts were sometimes found deep in the tumour, but otherwise the normal liver architecture completely disappeared. Large numbers of tumour cells could be seen in the portal and hepatic veins in the liver.

In two cases tumour cells were found filling the sinusoids of abdominal lymph nodes, and small groups of tumour cells were also found in the mesentery. The pulmonary arteries were often found packed with tumour cells but there was no extravascular spread. It was rare to find tumour cells elsewhere except in small numbers in the renal vessels.

The spleens showed hyperplasia, mainly of the pulp, and gross oedema when tumours were present. Degenerative changes were seen in the testes, myocardium and kidney. These appeared to be related to the prolonged use of the dye and not to the presence of tumour.

DISCUSSION.

The tumour cells bear a striking resemblance to Kupffer cells, though they appear to arise from the related cells in the periportal connective tissue rather than the true Kupffer cells of the sinusoids. The origin of Kupffer cells and the reticulum cells of the lymph nodes from related embryonic cells (Maximow, 1928) seems sufficient justification for calling the tumour a reticulum cell sarcoma. No record of the occurrence of a similar tumour in rats could be found, but an apparently identical spontaneously occurring tumour in mice has been described by Cloudman (1939) and Gorer (1946) under the name of reticulo-endothelioma. Metastases occurred in the mice in both liver and spleen, but were rare elsewhere except as intravascular emboli. Both the mice and the rats in the present experiment showed a severe anaemia, with tumour cells in the right heart blood but not the tail blood. The possibility of a Bartonella infection in the rats was considered as it has been induced by India ink (Rosenthal and Zohman, 1931). No definite evidence was obtained, though a typical attack occurred in a control rat following splenectomy. Both Cloudman and Gorer were able to transplant their mouse tumour, though it did not take readily even in pure line mice. It did not grow at the site of implantation, but appeared in the liver after a latent period of about 7 weeks. Twenty-four attempts to transplant the present rat tumours were unsuccessful, but in only 8 cases were the recipients kept alive over 8 weeks.

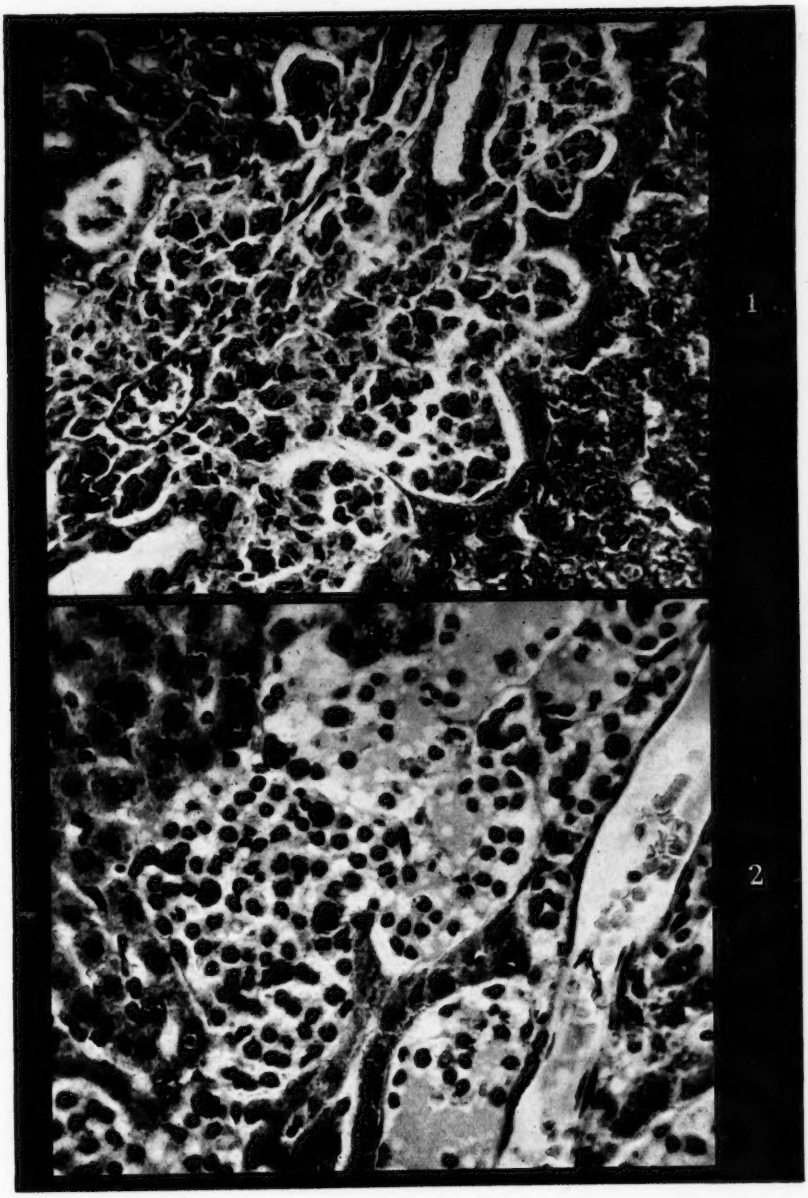
DESCRIPTION OF PLATES.

FIG. 1.—Portal tract after 84 days of staining showing scalloping. H. and E. × 370.

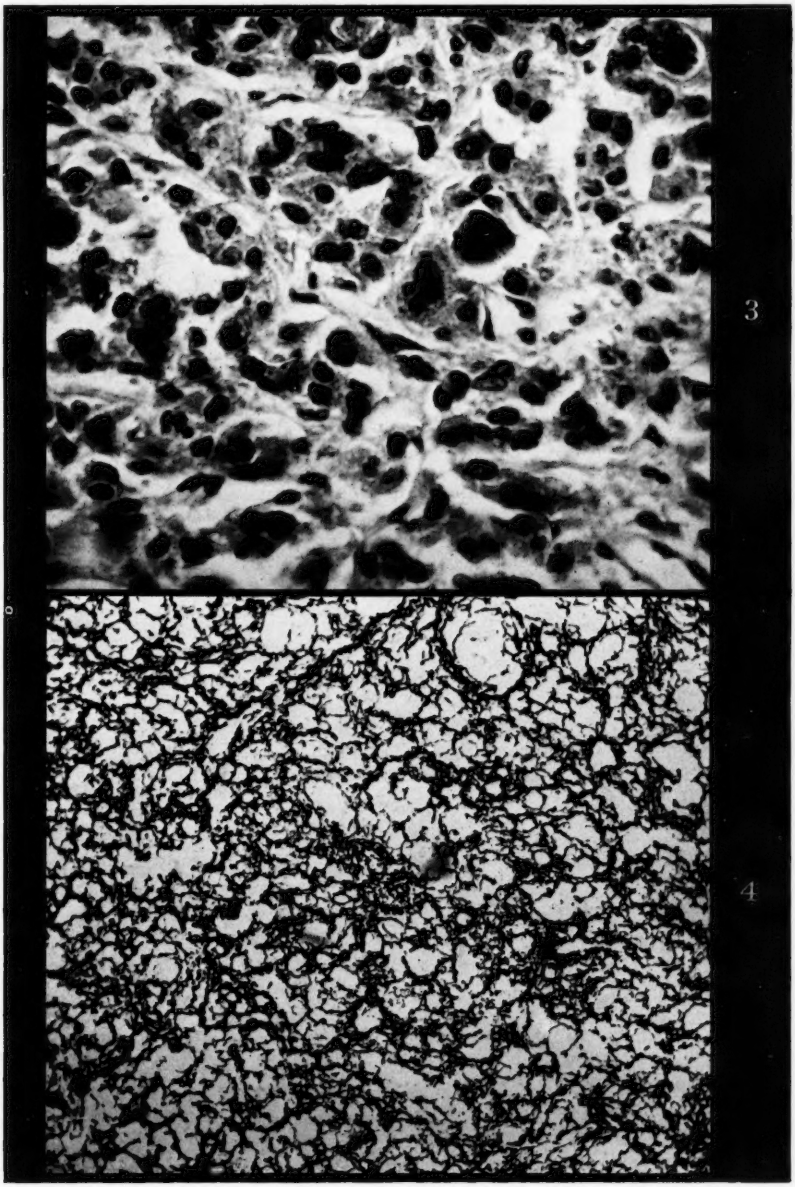
FIG. 2.—Portal tract on 196th day from a rat which died of reticulum cell sarcoma 41 days later. The cysts are separated by a single layer of parenchymal cells or by Kupffer cells only. H. and E. × 380.

FIG. 3.—Tumour showing irregularity of cells and some horse-shoe nuclei. H. and E. × 580.

FIG. 4.—Tumour showing heavy reticulin formation. H. and E. × 200.



Simpson.



Simpson.

Neither of the above workers reported changes in the portal tracts preceding tumour development, but they were described by Gillman *et al.* (1949) in their rats. The periportal histiocytic proliferation did not inevitably lead to tumour, as it was seen in well-developed form in biopsies at 180 and 280 days and at death after 447 days of staining in one rat without tumour formation. However, the impression remained that the more florid the picture at biopsy the more likely was rapid development of tumour. The formation of cysts seemed to be brought about by sudden expansion of the proliferating histiocytic masses allowed by necrosis of hepatic cells in the limiting lamina. Once cysts formed they persisted, and were seen in all specimens from that liver, even 180 days after cessation of the dye. The absence of fibrosis was one of the remarkable features of these livers.

The cause of the malignancy remains a matter for speculation. Vital stains and particulate matter taken up by the reticulo-endothelial system have rarely been associated with carcinogenesis. Schiller (1937) obtained fibrosarcomas at the site of injection in 4 out of 7 rats vitally stained for a year with Light green FS but this could not be confirmed (Kinosita, 1940). A much larger number of mice showed no tumours. Gillman *et al.* failed to obtain tumours in their rats with Congo red or Trypan red. It is possible that Trypan blue is itself a carcinogen, though it is not closely related to any of the known carcinogenic azo-dyes (Fig. 5).

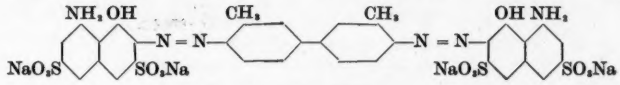


FIG. 5.—Trypan blue.

The commercial dye is not a pure substance and contains a variety of impurities, some coloured, as well as small amounts of the starting materials, *o*-tolidine and H-acid. There is no evidence that *o*-tolidine has any carcinogenic properties (Spitz, Maguigan and Dobriner, 1950). H-acid is 8-amino-l-naphthol-3 : 6-disulphonic acid. Hueper (1938) reported three retothelial sarcomas in a small group of mice injected with 2-amino-l-naphthol. Bonser, Clayson and Jull (1951) have shown the same substance to be carcinogenic when applied locally in the bladder. The diazo link in Trypan blue occurs at the 2-position, and reduction *in vivo* could produce an amino group in this position. This is theoretically possible, as Fuller (1937) showed that reduction of the diazo group occurs in the conversion of Prontosil to sulphanilamide. The concentration of the dye in the liver and the slow liberation of a carcinogen over a long period might then account for the site of tumour formation. None of the three vital dyes mentioned above is a naphthol derivative. No bladder changes were seen in the present rats, and the lymph nodes were only involved twice apparently by metastases. The Gillmans (J. Gillman, personal communication) found lymph-node tumours in 76 out of 193 rats in their series. Their tumours seem to resemble those seen in the present experiment rather than the common spontaneously occurring ileo-caecal lymphoma (Bullock and Curtis, 1930). Unfortunately Gillman *et al.* do not correlate the incidence of lymph-node tumours with liver tumours, though it is obvious that considerable overlapping must have occurred.

Variations in the amount or kind of impurities present in the I.C.I. dye, compared with those in the Grübler's stain used by the Gillmans, might account for some of the differences. While the Grübler stained rats lost appetite and weight

after each injection, those on the I.C.I. dye showed little ill effect. The remarkable development of hepatic cysts and the occasional occurrence of extensive hepatic necrosis reported by the Gillmans were not seen in the present experiment. The question of whether Trypan blue is itself responsible for the tumours therefore remains an open one.

SUMMARY.

A characteristic histiocytic proliferation in the portal tracts occurred in all rats stained with a commercial preparation of Trypan blue for more than 100 days.

Reticulum cell sarcoma of the liver occurred in 55 per cent of the rats surviving 150 days of staining. The minimum carcinogenic dose given was 110 mg. of dye.

There is no evidence as to which component of the dye is the cause of the changes.

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TWO HEAT-LABILE FACTORS IN NORMAL SERA WHICH NEUTRALIZE VARIOLA VIRUS.

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THE means by which the immune or the non-immune host attempts to dispose of an infecting virus are obscure. Studies of the fate of inoculated virus within the body of the host throw little light on the actual disposal mechanisms, and the problem is usually approached by making virus neutralization tests in which a mixture of serum from the test animal and virus is inoculated into a suitable indicator animal. In such tests it is customary to heat-inactivate the serum before making the mixtures so that only the heat-stable specific antibody is subjected to test. Most of our knowledge of humoral immunity to virus infection has been derived from such tests.

In contrast to the vast amount of study which has been devoted to heat-stable antibodies, few workers have investigated the rôle of heat-labile serum factors in the immunology of virus infections.

Factors with the capacity to enhance specific neutralization have been described. Thus Gordon (1925) was able in one experiment to demonstrate a marked reduction in virus neutralizing power when a fresh antivaccinial rabbit serum was heated, and further that the loss could be restored, the addition of fresh guinea-pig serum increasing the neutralization 100-fold. However, in two other samples of rabbit serum which Gordon tested, heating did not lessen the neutralizing activity ; Gins (1916) also failed to demonstrate enhanced neutralization with unheated serum. Mueller (1931) showed that the neutralizing capacity of fowl antibody to Rous sarcoma could be intensified and the loss of titre of the heated antiserum restored by the addition of fresh guinea-pig serum. Meyer (1941, cited by Morgan, 1945) showed that the phagocytosis of psittacosis virus was greater with fresh than with old immune serum, and that the effect of heated antiserum was augmented by adding " complement."

Morgan (1945) and Whitman (1947), both working with Western equine encephalomyelitis virus, showed that fresh immune human serum stored at — 70° possesses far greater neutralizing activity than heated serum or serum kept at 4°. By adding fresh guinea-pig serum Morgan could restore the lost activity of sera kept at 4° but not of heated sera. Whitman, however, could restore the lost activity to old and to heated sera by adding fresh normal serum from guinea-pig, monkey or man. Working with dengue virus, Sabin (1950) found that normal guinea-pig, rabbit or human sera could restore the loss incurred by heating an immune serum.

Dozois, Wagner, Chemerda and Andrew (1949) working with encephalomyelitis rabbit antiserum claim to have shown that the potentiating factor in normal rabbit

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serum involved the 2nd, 3rd and 4th component of haemolytic complement. Leymaster and Ward (1949) using mumps virus showed that the chick embryo-protective titre of a mumps antiserum (human or monkey) was markedly reduced by heat inactivation or by absorption with an antigen-antibody precipitate, and that this lost activity could be restored by adding unheated normal human serum but not by heated.

The following workers, however, have failed to demonstrate any potentiating effect with " complement ": Howitt (1934) using horse antiserum, guinea-pig complement, and WEE virus, and Florman and Enders (1942) using rabbit antiserum, fresh normal rabbit serum as complement and vaccinia virus in roller tube cultures of mononuclear cells. Fox (1949) working with *Rickettsia orientalis* likewise found no detectable effect on the degree of neutralization when immune guinea-pig serum was heated, nor when fresh guinea-pig serum was added to the system containing heated immune serum.

In addition to the power of fresh normal sera to potentiate specific virus neutralization by heated immune sera, it is known that normal serum itself may be capable of suppressing virus growth. In the case of poliomyelitis the factor responsible is heat-stable (Jungeblut, 1934). Jungeblut and Engle (1934) showed that the presence of such a heat-stable factor might play some part in determining the resistance of monkeys to poliomyelitis. For the most part, however, the virus inhibitors observed in normal sera have been heat-labile. Thus the viruses of Newcastle disease and of influenza may be inhibited in chick embryos by a heat-labile factor in normal sera (Ginsberg and Horsfall, 1949 ; Howitt, 1950 ; Bang, Foard and Karzon, 1950, 1951 ; Francis, Salk and Brace, 1946 ; Burnet and McCrea, 1946 ; McCrea, 1946 ; Smith and Westwood, 1949). Similar findings have been reported with vaccinia virus (Douglas and Smith, 1930 ; Briody, Ledinko and Stannard, 1951).

Psittacosis virus (Utz, 1948) and the viruses of St. Louis, Japanese B and Russian Far East encephalitis in mice are apparently inhibited by a lipoid fraction of normal sera from several species (Casals and Olitsky, 1947, 1948).

In the recent paper of Briody *et al.*, cited above, it is reported that fresh guinea-pig serum contains a heat-labile factor which inhibits the growth of vaccinia virus on the chorio-allantoic membrane. This factor is absent from mouse sera. It was apparently also present, though in less degree, in the fresh normal rabbit sera which these authors tested.

The variable results recorded by different workers suggest differences in the susceptibility of viruses to heat-labile factors in normal serum and to differences in the sera from different animals, but in addition the nature of the tests and of the animal species used in them probably influence results.

EXPERIMENTAL.

The studies reported here with smallpox virus were undertaken to discover whether fresh normal human sera were all alike in their action on smallpox virus, and whether complement or some similar factor might play a part in the specific neutralization of smallpox virus by immune sera. The ease with which smallpox virus can be grown on the chorio-allantoic membrane, the quantitative estimation of infective virus by pock counts, the lack of haemolytic complement in chick embryos (Polk, Buddingh and Goodpasture, 1938) and their inability to form antibody seemed to offer a suitable method for studying these problems.

Materials and methods.

The variola virus used was a purified elementary body suspension from a chick-embryo-propagated strain isolated in this laboratory from a smallpox case. Virus from the fifth egg passage was used, and a concentration chosen for the tests such as would give about 150 lesions per egg in control mixtures. The vaccinia virus was a rabbit-adapted strain. The cowpox virus was a rabbit-adapted strain which had been transferred to eggs and passed 10 times on the chorio-allantoic membrane.

The strain of herpes simplex virus was isolated from labial herpes and had had 14 egg passages. The normal human sera were obtained by venepuncture from unvaccinated young adults who had not had smallpox. The immune human sera came from adults who had had several successful vaccinations, the most recent being within a year. Sera from normal animals which had not been exposed to infection with viruses of the pock group were also used. They were obtained by venepuncture, cutting the carotid artery of stunned animals, or cardiac puncture under ether anaesthesia.

Blood samples were allowed to clot for 1 hr. at 37°, then transferred to a refrigerator for 1–2 hr., the serum separated and transferred in small quantities to glass ampoules which were sealed and stored in dry ice-ethyl alcohol mixture. A few were stored at about — 50° in dry ice alone. When necessary sera were heat inactivated at 58° for 30 min. The diluting fluid used throughout was 10 per cent broth in saline containing 100 units/ml. penicillin.

Neutralization tests in eggs were made by adding 0·4 ml. virus, suitably diluted, to 0·1 ml. of the serum to be tested and 0·1 ml. broth saline. Where two sera were used in the same mixture the second one replaced the broth saline. After the mixtures had stood at room temperature for 40–60 min. each was distributed on the chorio-allantoic membrane of five to eight 11–13-day–old chick embryos. Lesions were counted after 3 days' incubation at 36°. After discarding dead embryos and occasional badly scarred membranes the average number of lesions per membrane was determined for each mixture, and from this the percentage survival was calculated, assuming 100 per cent survival in the control mixtures of virus and heated normal serum. The survival rate in such control mixtures did not differ from the survival rate when virus was mixed with broth saline alone. Variations from these techniques are recorded in the text.

TABLE I.—*The Inhibition of Smallpox Virus by Unheated Normal Guinea-pig Sera.*

	Serum.	Pock counts on individual eggs.	Mean count per egg.	Per cent survival.
1	Unheated	16, 16, 31, D, D	21	16
1	Heated	99, 134, 165, 176, 187	152	116
2	Unheated	8, 11, 29, 29, 51	26	20
2	Heated	69, 100, 125, 147, D	110	84
3	Unheated	32, 40, 48, 55, 80	51	39
4	,,	12, 23, 45, 68, D	37	28
5	,,	14, 27, 33, 80, (Scarred)	38	29
6	,,	26, 32, 44, 90, 44	47	36
Normal human (M).	Heated	54, 96, 137, 200, 207	131	100
,, ,, (S).	,,	109, 116, (Scarred), D, D		

Control: Average count in mixtures containing heated normal human serum = 131 (i.e., 100 per cent).

(Scarred) = Scarred membrane which was discarded.
D = Dead embryo. Membrane discarded.

RESULTS.

The Occurrence of Inhibiting Factors in Normal Human and Animal Sera.

Table I shows the result of a typical experiment in which six normal unheated guinea-pig sera were tested for neutralizing power against variola virus. It will be seen that all effect significant neutralization, experience having indicated that the neutralization of 50 per cent or more of the virus is significant when 5 or 6 eggs are used for each mixture. The inhibiting power of the sera (Nos. 1 and 2) was destroyed by heating at 58°.

Table II shows the pooled results of tests on sera from different host species.

TABLE II.—*The Occurrence of Inhibiting Factor for Smallpox Virus in Unheated Normal Sera.*

Fresh normal serum from—	Number of animals tested.	Number showing significant neutralization of smallpox virus (i.e., > 50 per cent).
Guinea-pig	19	14
Rabbit	15	12
Man	12	5

In addition to these experiments with variola virus, a few preliminary tests were made with herpes simplex and cowpox viruses using the same technique. The results have been similar to those with variola virus.

A further experiment using vaccinia virus was made by inoculating virus-serum mixtures intradermally into a rabbit and also into a guinea-pig. The lesions in both cases were smaller when the virus was mixed with unheated than when mixed with heated normal human serum.

A Heat-labile Factor in Normal Human and Animal Sera which Potentiates Neutralization of Variola Virus by Specific Heat-stable Antibody.

It was found that if a fresh unheated normal human serum, chosen because it lacked any significant inhibiting power when tested by itself, was added to a mixture of virus and heated immune serum, the extent to which the virus was neutralized was greatly increased. If, however, the normal serum was first inactivated by heat, this potentiating power was lost. Table III shows a typical

TABLE III.—*Smallpox Virus Neutralization by a Heated Immune Serum, Potentiated by Fresh Normal Serum.*

Human sera used.	Pock counts on individual eggs.	Mean.	Per cent survival.
Heated normal + saline	107, 108, 267, 120, 179, 236,	169	100
Unheated normal + saline	82, 100, D, 110, 192, 205,	138	
„ immune + saline	1, 1, D, 5, 9, D,	4	3
Heated immune + saline	15, 37, 97, 40, 46, 72,	51	33
„ „ + heated normal	16, 29, D, 50, 58, 86,	48	31
„ „ + unheated normal	7, 10, D, 12, 15, D,	11	7

example of such an experiment. The small difference between the mean counts of heated and unheated normal sera is probably the result of chance, since in another experiment with this same serum the difference was in the reverse direction. However, it appears from our limited observations that in guinea-pig, rabbit and human sera, the natural antibody-like factor mentioned in the preceding section varies in activity, some sera possessing much and others being for practical purposes without such activity.

It will be seen from Table III that an immune serum which has been stored at low temperature loses much of its neutralizing power when heated, and that this power can be partially restored by adding unheated normal but not by heated normal human serum. The failure to achieve complete restoration has characterized all our experiments of this type. There seem to be three possible causes: the immune serum itself may have possessed some heat-labile natural antibody-like factor; it may have possessed heat-labile potentiating factor, more efficient than that provided by adding unheated normal serum from another individual; or some part of the specific acquired antibody may be heat-labile.

It will be seen from Table II that 5 guinea-pig, 3 rabbit and 7 human sera had no significant inhibitory effect on variola virus. Of these, none of the guinea-pig sera, one of the rabbit sera—the only one tested—and all the human sera potentiated neutralization by heated specific human immune serum. The properties of the potentiating factor and of its relation to the naturally occurring inhibiting factor and to haemolytic complement are further considered in the following section.

The next experiment was designed to show that potentiating factor occurs also in sera possessed of natural inhibiting factor. This effect cannot be demonstrated in the direct manner as in Table III, since the potentiating effect is masked by the natural antibody-like effect. Table IV shows the result of an experiment using two

TABLE IV.—*Demonstration of Potentiating Factor in a Normal Human Serum (B) Possessed of the Natural Antibody-like Factor.*

Sera in mixtures.	Pock counts.	Mean.	Per cent survival.
Heated normal B + heated normal B	51, 106, 135, 163, 190	129	100
„ „ B + unheated normal B	30, 32, 36, 41, 83	44	34
Unheated normal B + unheated normal B	(0), 11, 13, 23, 30, 39	23	18
Immune C (heated) + heated normal B	9, 12, 26, 42, 83, 99	45	35
„ „ „ + unheated normal B	0, 1, 1, 2, 3, 4	2	2
„ M „ + heated normal B	13, 14, 68, 69, 77, 87	55	43
„ „ „ + unheated normal B	0, 1, 2, 3, 8, 17	5	4
„ „ „ + immune C heated	9, 21, 43, 44, D, D	29	22

(0) = Badly developed membrane (discarded).

heated immune sera of about equal strength, combined in various ways with a single normal human serum which possesses considerable natural antibody-like effect.

It will be seen that approximately the same degree of virus neutralization is effected when heated immune serum M, heated immune serum C or unheated

normal serum B are mixed with an equal volume of heated normal serum B. When the two heated immune sera were combined, the proportion of virus surviving was almost halved, and similarly when two volumes of the unheated normal serum B were combined, the survival rate was almost halved. But when one volume of either of the immune sera was mixed with one volume of the unheated normal serum, the survival rate was reduced to less than 5 per cent. It appears from this result that the normal serum possesses, in addition to its natural antibody-like effect, considerable power of potentiation. It therefore seems probable that all normal human sera possess potentiating factor.

Some Properties of the Two Heat-labile Components of Normal Human Sera.

The possibility was considered that the natural antibody-like effect shown by some unheated normal human sera might merely represent an additional property of the potentiating factor which is present in all, only becoming evident as an inhibiting factor when present in excess.

The next experiment (Table V) shows that this is not so : normal serum E was known to be completely lacking in natural antibody-like factor, whereas normal

TABLE V.—*The Potentiation of Specific Neutralization of Smallpox Virus by Dilutions of Two Unheated Normal Sera.*

Human sera and dilutions used.	Pock counts.	Mean.	Per cent survival.
Heated normal E + saline	Scar, 54, 57, 87, 115,	78	100
„ immune + „	D, 5, 11, 12, 23,	13	17
Unheated normal E + saline . . .	34, 64, 74, 96, 100	74	100
„ „ A + „ . . .	D, 11, 12, 15, 31	17	22
Heated immune + unheated normal E 1/1	0, 0, 1, 2	1	1
„ „ + „ „ E 1/5	0, 1, 4, 8	3	4
„ „ + „ „ E 1/25	0, 3, 5, 5	3	4
„ „ + „ „ A 1/1	1, 3, 3, 4	3	4
„ „ + „ „ A 1/5	1, 6, 8, 10	6	8
„ „ + „ „ A 1/25	2, 14, 15, 18	12	16

serum A had this inhibiting factor to the extent that it allowed less than 25 per cent of virus to survive when tested by itself. In contrast, however, the potentiating power of serum E is very good even at a dilution of 1/25, whereas that of serum A, although good when tested undiluted, was waning at 1/5 and was not demonstrable at a dilution of 1/25. It appears therefore that these two serum factors are independent of each other.

Relation to haemolytic complement.—Table VI shows the lack of correlation between haemolytic complement titre and the presence of natural antibody in 7 normal human sera. The presence of potentiating factor is likewise not correlated with the haemolytic complement titre, since it has been shown to be absent from a

TABLE VI.—*The Lack of Correlation between Haemolytic Complement Titre and the Presence of Natural Antibody in Seven Normal Human Sera.*

	Sera tested.						
Serum No.	1	2	3	4	5	6	7
Per cent virus neutralized .	84	0	54	30	15	59	73
Complement titre . .	1/20	1/20	1/20	1/10	1/20	1/10	1/20

guinea-pig serum with a complement titre of 1/30, and yet to be very active in a human serum in which complement could not be detected at a dilution of 1/10.

The two factors seem to be distinct from intact haemolytic complement. Fractionation of complement was not undertaken.

Stability of the two factors.—Both factors have been shown repeatedly to be destroyed by heating for 30 min. at 58°. Ten minutes at 59° seemed equally effective. In one experiment the potentiating factor was only slightly affected by overnight storage at 4° or even at room temperature, and the natural antibody-like factor in another normal human serum seemed to be quite unaffected by the same treatment.

Rapidity of action of the two factors.—Table VII shows that the natural antibody-like factor acts very quickly, a few seconds sufficing for almost maximal effect. There was only slight improvement in neutralization after 2 hr. contact with virus.

TABLE VII.—*Rate of Neutralization of Variola Virus by "Natural Antibody."*

Sera (human).	Time of contact before inoculating mixtures into eggs.	Pock counts.	Mean.	Per cent survival.
Heated normal	30 min.	(4), 51, 66, 98	72	100
Unheated normal	10 sec.	D, 15, 18, 45	26	36
„ „	30 min.	4, 11, 22, 43	20	28
„ „	120 „	11, 12, 15, 31	17	24

In another experiment it was found that adding the potentiating factor immediately before inoculating the eggs to an immune serum/virus mixture which had stood at room temperature for the usual 40–60 min. produced as good neutralization as when the normal serum potentiator was incorporated in the virus-immune serum mixture from the start. Both factors appear therefore to achieve their maximal effect very quickly.

The effect of dilution on the two factors.—Only a few observations have been made, but it appeared that the natural antibody was weak or absent at a serum dilution of 1/5. The potentiating factor on the other hand may still be quite active at a dilution of 1/25 (Table V).

Normal human and rabbit sera, both of which possessed potentiating factor, did not in a dilution of 1/10 enhance the vaccinia haemagglutinin inhibitory action of a human immune serum.

DISCUSSION.

The significance of these two factors in the defence mechanism of the host is unknown. Recently Briody *et al.* have brought forward evidence to suggest that natural antibody to vaccinia virus present in some species may play a part in determining species susceptibility to infection. It may well be that the variable level of the natural antibody-like factor in normal human sera plays some part in determining the severity of smallpox in the unvaccinated. It is probably unimportant in those who have acquired antibody.

The potentiating factor on the other hand is probably important in disposing of invading virus, since it greatly enhances the activity of immune sera. We have not attempted to compare the levels of potentiating factor in normal sera, nor do we know whether these levels are constant in the same individual. We have not

among the small number of human sera examined found any which lacked this factor. It was even present in a child dying of generalized vaccinia through apparent congenital inability to form antibody to the virus.

The failure to restore completely the lost activity of a heated immune serum by the addition of fresh normal serum raises the possibility that some part of the specific acquired antibody may be heat-labile.

In comparing our findings with variola virus with those of Briody *et al.*, who found vaccinia-inhibiting power in all guinea-pig sera examined, a quarter of the guinea-pigs we tested lacked variola-inhibiting factor. Also the levels of inhibiting power which we encountered were apparently lower, although this difference may have arisen from the use of different test virus. The inhibitory activity observed in guinea-pig sera by these workers seemed to be more labile than that found by us in normal human sera against variola virus. They make no mention of a potentiating factor for vaccinia virus. We were unable to demonstrate such in the guinea-pig sera which we tested against variola virus.

SUMMARY.

Normal human sera either fresh or stored at — 50 to — 75° contain a factor which potentiates the specific neutralization of variola virus by heated human immune serum.

In addition some normal human sera also contain a factor which acts like a natural antibody in that it can by itself neutralize variola virus.

Both factors are heat-labile.

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HAEMAGGLUTINATION WITH THE VIRUS OF MURRAY VALLEY ENCEPHALITIS (MVE).*

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UNTIL the last few years attempts to obtain haemagglutinins from the "neurotropic" viruses have been unsuccessful, but recently several viruses of this group have been shown to produce haemagglutination under very strictly defined conditions, the method of preparation of the haemagglutinin, ionic strength and pH of the reaction medium, type of red cell used and temperature of incubation being the chief controlling factors.

It is now well established that specific haemagglutinin can be obtained from the EMC complex of viruses. Agglutination of sheep erythrocytes by Col SK and Col MM was reported by Hallauer in 1947 (Hallauer 1949). Olitsky and Yager (1949) confirmed this work, and in addition found that EMC and Mengo encephalomyelitis viruses were capable of agglutinating sheep erythrocytes. Later Verlinde and de Baan (1949), Bremer (1951) and Gard and Heller (1951) extended these observations on agglutination of sheep erythrocytes by the EMC viruses.

Among the poliomyelitis-like viruses, only the GD VII strain of mouse encephalomyelitis has so far yielded a specific haemagglutinin. Lahelle and Horsfall (1949) found that suspensions of mouse brain infected with GD VII would specifically agglutinate human Group O cells in the cold and that this agglutination was reversible at 37°. These workers were unable to prepare haemagglutinins from the FA and TO strains of mouse encephalomyelitis. Attempts to demonstrate specific haemagglutination with the poliomyelitis viruses themselves have so far proved unsuccessful (Sabin, 1951).

Viruses responsible for the arthropod-borne encephalitides have recently been shown by Sabin and Buescher (1950) and Sabin (1951) to produce haemagglutination. Specific haemagglutination of newly hatched chick red cells has been produced by Japanese B encephalitis, St. Louis encephalitis and West Nile viruses, and of sheep erythrocytes by Russian Spring Summer encephalitis virus. All of these haemagglutinins have very exacting requirements. The viruses of Eastern equine and Western equine encephalitis have so far failed to yield haemagglutinin.

A new member of this group of viruses, namely Murray Valley encephalitis, has recently been isolated by French (1952) in Victoria, Australia. This virus appears to be fairly closely related to Japanese B encephalitis virus, and it therefore seemed probable that methods similar to those used by Sabin and Buescher for the production of Japanese B haemagglutinin might be successful with MVE.

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This proved to be the case. However, a simpler method of preparation, which yields high titre haemagglutinin for both MVE and Japanese B, will be described. Furthermore, red cells from pigeons (a much more convenient source of erythrocytes than newly hatched chickens) have proved agglutinable by both Japanese B and MVE viruses.

MATERIALS AND METHODS.

Haemagglutination with preparations of MVE-infected material will take place only within a narrow range of conditions. The optimum conditions for the haemagglutination reaction have been determined experimentally, and a standardised technique has been adopted for testing the various haemagglutinin preparations to be described.

. *Diluent.*—Throughout the entire course of the experiments the diluent used was that employed by Sabin and Buescher, namely 0·01M phosphate buffer made up in 0·9 per cent saline, at pH 6·5–6·75.

Cells.—Chicks were bled from the heart into citrated saline during the first 24 hr. after hatching. After washing, the cells were made up to 1·5 per cent in physiological saline.

Volumes.—Doubling dilutions of 0·25 ml. of haemagglutinin were prepared in phosphate buffered saline. One drop of 1·5 per cent chick cells was added to each dilution, giving a final cell concentration of about 0·25 per cent.

Technique.—The bulk solution of buffered saline was kept at 4°. Buffer in volumes of 0·25 ml. was pipetted into tubes, which were always left at 4° for at least 15 min. before the preparation of haemagglutinin dilutions. These were prepared at room temperature, and not longer than 5 min. was allowed to elapse between the start of preparation of dilutions and the addition of cells. The racks were then returned to the refrigerator, and after about 1 hr. at 4° agglutination was recorded by reading the pattern of the agglutinated cells (Salk, 1944).

Preparation of Haemagglutinin.

Throughout this investigation the virus used for the production of haemagglutinin was MVE (Strain 1) in its 13th mouse passage. Two attempts to prepare haemagglutinin from this material inoculated on the chorioallantoic membrane have failed. However, three methods of preparation from infected mouse brain have been successful.

(a) *Ultracentrifugation of 21-day mouse brains.*—Following exactly Sabin and Buescher's (1950) method for the production of Japanese B haemagglutinin, saline suspensions were prepared from brains of 21-day mice inoculated intracerebrally with MVE. Two such preparations, after storage for about 3 weeks at 4°, gave haemagglutinin titres of from 80 to 640, titres much lower than those obtained by Sabin and Buescher with Japanese B encephalitis haemagglutinin.

(b) *Ultracentrifugation of 5- to 10-day mouse brains.*—Since MVE multiplies to higher titres in the brains of suckling mice than in the brains of 21-day mice (Macdonald, 1952), this technique was used on suspensions of brains from 5- to 10-day mice inoculated intraperitoneally with MVE. Three such preparations normally gave haemagglutinin titres of 1280 to 2560. Two of these preparations gave low titres (about 20 to 80) when tested within 7 days of preparation, but both subsequently developed titres up to 2560 after storage at 4° for more than one

week. This enhancement of titre on storage was also noted by Sabin and Buescher (1950).

(c) *Light centrifugation of 5- to 10-day mouse brains.*—The following method was finally adopted as the routine technique for preparation of MVE haemagglutinin: Five- to 10-day-old mice were inoculated by the intraperitoneal route with about 100,000 LD_{50} of MVE. When showing symptoms (4–5 days later) the mice were killed with chloroform, the brains were removed, washed with saline and ground with alundum. A 20 per cent w/v suspension was prepared in 0·9 per cent saline and centrifuged at 2500 r.p.m. for 10 min. The supernatant was pipetted off into ampoules which were quickly frozen in CO_2 ice/alcohol mixture and stored at — 70°.

Five such preparations have been made and all have given high haemagglutinin titres, varying from 5120 to 40,960. Very occasionally, with some cells, titres as low as 640 have been obtained. High titres are obtained only when the above method of preparation is closely followed. There has been no diminution in titre after 4 months' storage at — 70°. Preparations of haemagglutinin made from brains extracted with distilled water gave lower titres, while preparations from brains extracted with broth at pH 7·6 gave irregular results.

Japanese B haemagglutinin has been prepared in exactly the same manner from 5- to 10-day mouse brains infected with Japanese B virus. This haemagglutinin gave titres of 5120–10,240 when tested with standard baby chick cells. Such titres are comparable to those obtained by Sabin and Buescher with ultracentrifuged preparations.

Specificity of MVE haemagglutinin reaction.

Preparations made in the same way from the brains of both normal baby mice and herpes-infected baby mice have never shown any trace of haemagglutinating activity. Further, haemagglutination can be inhibited by sera known to contain antibody to MVE.

The Haemagglutination-Inhibition Test.

The haemagglutination-inhibition (HI) technique is complicated by the fact that all sera so far tested, *i.e.*, sera from fowls, guinea-pigs, rabbits, monkeys and humans, contain an inhibitor to MVE haemagglutinin active to titres which are sometimes sufficiently high to mask the action of specific antibody in inhibiting agglutination. All or most of the non-specific inhibitor can be removed by shaking sera with lipoid solvents such as chloroform, toluene or ether. This treatment apparently leaves specific antibody unaffected. Sabin and Buescher (1950) report a similar experience with Japanese B.

In the HI tests to be described, rabbit and fowl sera were used. Antisera against MVE were prepared by inoculating 2 rabbits and 2 fowls with MVE, preinoculation bleeds being obtained from each animal. Neutralization tests were carried out with post-inoculation bleeds from all 4 animals. Both fowl sera contained a high level of neutralizing antibody, and both rabbit sera contained a low level of neutralizing antibody. By means of the complement-fixation test it was shown that both post-inoculation rabbit sera contained complement-fixing antibody against MVE. Miss M. Eagle, of this Institute, using the indirect com-

plement-fixation test, showed that both post-inoculation fowl sera contained complement-fixing antibody.

In the HI test all sera were extracted with ether, 0·25 ml. undiluted serum being shaken up with 0·75 ml. ether. When the serum had separated out it was pipetted off from the ether and a 1/10 dilution was prepared in phosphate buffered saline. These dilutions were heated to 62° for 15 min. and diluted out for the HI test.

The test was carried out as follows : Doubling dilutions of serum were prepared in phosphate buffered saline in volumes of 0·25 ml. To these, 0·25 ml. of a suspension containing 8 to 16 agglutinating doses of MVE was added and finally 2 drops of standard 1·5 per cent chick cells, giving a final cell concentration of about 0·25 per cent. Table I shows a representative HI test.

TABLE I.—*Representative Haemagglutination-Inhibition Titration.*

Red cells : from chick < 24 hr. old.
Haemagglutinin : lightly centrifuged MVE-infected brains from 5- to 10-day-old mice.
8–16 agglutinating doses used.

Preparation.	Type of serum.	Serum dilutions. 20.	40.	80.	160.	320.	640.	1280.	Titre.
IV	Rabbit I	++	++	++	++	++	++	++	<20
	,, II	++	−	−	−	++	++	++	480
	Hen I	++	++	++	++	++	++	++	<20
	,, II	++	++	−	−	−	++	++	960

Rabbit I and Hen I : pre-inoculation bleeds.
Rabbit II : contains complement-fixing antibody and low level of neutralizing antibody to MVE.
Hen II : contains complement-fixing antibody and high level of neutralizing antibody to MVE.

Table II shows that satisfactory haemagglutination inhibition has been obtained with all haemagglutinin preparations tested.

TABLE II.—*Specificity of MVE Haemagglutinin Preparations.*

Red cells : from chicks < 24 hr. old.
Haemagglutinin : 8–16 agglutinating doses.

Haemagglutinin preparation.	HI antibody titre. Rabbit 227 I (pre-inoculation).	Rabbit 227 II (CF and neutralizing antibody).
Ultracentrifuged	<20	2000
Lightly centrifuged I	<20	2000
,, ,, II	<20	800
,, ,, III	<20	1000
,, ,, IV	<20	480
	Rabbit 226 I (pre-inoculation).	Rabbit 226 II (CF and neutralizing antibody).
,, ,, V	<20	400

Finally, human and rabbit sera known to contain neutralizing antibody to herpes virus and a guinea-pig antiserum to mouse encephalomyelitis virus have failed to give haemagglutination-inhibition when tested under similar conditions against two separate MVE haemagglutinins prepared from baby mouse brain.

Other red cells.

Erythrocytes from other animals have been tested with MVE haemagglutinin. Human Group O cells and ferret cells have failed to give agglutination, and horse cells have given very irregular results.

Irregular results have been obtained by using erythrocytes from chicks older than 24 hr., and cells from adult fowls (including fowls producing vaccinia-sensitive cells) have, with one exception, failed to give any haemagglutination.

Pigeon cells have, however, reacted quite as well as cells from newly hatched chicks; when tested with both MVE and Japanese B haemagglutinins they have agglutinated to titres comparable to those obtained using standard baby chick cells. Pigeon red cells do not agglutinate in the presence of normal baby mouse brain or herpes-infected mouse brain suspensions. Furthermore, agglutination of pigeon cells by MVE haemagglutinin can be prevented by MVE antisera (see Table III).

TABLE III.—*MVE Haemagglutinin and Pigeon Cells.*

Haemagglutinin: 8–16 agglutinating doses.

Haemagglutinin.	Serum.	Serum dilutions. 20.	40.	80.	160.	320.	640.	1280.	2560.	Titre.
Lightly centrifuged V	Rabbit 226 I	++	++	++	++	++	++	++	++	<20
	„ 226 II	++	−	−	−	−	−	tr.	++	2000
	„ 227 I	++	++	++	++	++	++	++	++	<20
	„ 227 II	++	−	−	−	−	++	++	++	480

Rabbit 226 I and Rabbit 227 I: pre-inoculation bleeds.
Rabbit 226 II and Rabbit 227 II: contain complement-fixing and low level of neutralizing antibody to MVE.

Since pigeons are a much more convenient and more readily available source of red cells than newly hatched chicks this finding is of some importance, particularly if the haemagglutination-inhibition reaction is to be used as a diagnostic test for the presence of MVE or Japanese B infection.

SUMMARY.

A method for preparing MVE virus haemagglutinin by light centrifugation of brains of infected baby mice is described.

Japanese B encephalitis virus haemagglutinin has been prepared by a similar method.

Pigeon and newly hatched chick erythrocytes have been found to be agglutinable by MVE and Japanese B-infected baby mouse brain suspensions.

Evidence is presented that the haemagglutinin described is a specific product of the MVE virus infection.

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THE EFFECT OF CERTAIN NON-SPECIFIC FACTORS ON THE PRODUCTION OF ANTITOXIN.

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IT is well known that an increase in the response of animals to an injection of toxoid may be brought about by the addition to the toxoid of such substances as alum, aluminium hydroxide or phosphate, turpentine or toluol (see Glenny, Pope, Waddington and Wallace, 1926). The increased response must be regarded as due to an enhancement of the specific stimulus. Factors such as bleeding, or injections of other antigens, which we will call non-specific, may also have an effect both on rapid antitoxin production after secondary stimulation, and on the slow continued production that continues for months or years after the last specific stimulus.

Glenny, Buttle and Stevens (1931) visualised the effect of injection of antigen into a normal animal as a process of continual stimulation by lessening quantities of antigen, acting on tissues whose power of response is rapidly increasing. Barr (1950) suggested that two stages might be necessary in the development of antitoxin, some of the injected antigen producing or increasing potential immunity, and the remainder acting upon already sensitised cells to produce antitoxin. If some of the antigen remained to act on the cells thus sensitised, antitoxin would be produced after primary stimulation. After second and subsequent injections part of the injected antigen might increase existing potential immunity, and some might stimulate cells already sensitised, so that rapid antitoxin production ensued.

It was shown by Barr and Glenny (1947) that considerable active antitoxin production continued in hyperimmunised horses for long intervals after the last injection of specific antigen, and an instance was given of a horse whose tetanus antitoxin titre, 13 months after the last injection of toxin, was 87·9 per cent of that recorded when injections ceased. We also showed that a rise in titre (the "delayed response") occurred in certain guinea-pigs after the initial rapid fall from the peak of a secondary response following an injection of diphtheria toxoid. It was suggested that the two types of response, the secondary and delayed responses, might be explained by differences in the degree of sensitisation of parts of the immunity-producing mechanism. Some parts of this mechanism would become sensitised in such a way that a further injection would produce a rapid rise in titre, *i.e.*, a secondary response. Other tissues requiring a bigger stimulus or a longer rest would not produce antitoxin rapidly, but slowly and continuously. It was thought improbable that the combination of the secondary and delayed responses occurring in guinea-pigs could represent the result of a gradual primary response with an independent secondary response superimposed. In a later publication (Barr and Glenny, 1949) it was suggested that continued production

in the absence of stimulation might be due to reproduction either of cells containing replicas of the antigen, or of cells so modified that antitoxic globulin was formed as a normal product of metabolism.

Holt (1950) considered that the antitoxic titres reached in a secondary response were composed of the initial titre before injection, the secondary response leap (*i.e.*, the rapid rise) and a contribution due to the new injection of antigen. He considered from the results of his experiments that the antitoxin showing itself in the leap was already present in the animal as reserve or stored antibody, and visualised any new contribution apart from the leap as being produced slowly in the manner of antitoxin production after primary stimulation. According to this hypothesis, rapid production of antibody would never occur, and the functions of immunisation would be (1) the establishment or increase of continued production, and (2) the storing of antibody which is released when further specific stimulation is given. Barr and Llewellyn-Jones (1951) pointed out however that secondary response leaps of considerable magnitude frequently occurred after a second injection of antigen into guinea-pigs that had at no time shown any detectable circulating antitoxin after the first injection. The existence of potential immunity in primarily stimulated animals with no detectable antitoxin was first demonstrated by Glenny and Allen (1922). Further evidence of its occurrence has been obtained in man and in horses by Lemetayer, Nicol, Girard, Corvazier and Cheyroux (1951). Barr and Llewellyn-Jones found it difficult to visualise the accumulation and storage of antitoxin in the bodies of such animals, when none had ever been detected in the circulation before the second injection of antigen. It is also difficult to explain on the storage hypothesis the very rapid and enormous rises in titre that occur at some stages in the hyperimmunisation of horses.

After secondary stimulation of an animal with good basal immunity, and also at the end of a course of hyperimmunisation with toxin or toxoid, a rapid fall in antitoxic titre occurs after the peak of the response, and a subsequent abrupt levelling in the titre may occur. This suggests that the rate of antitoxin production reaches a maximum, then suddenly declines and soon reaches an almost constant rate, at a time when the actual titre is still falling: this fall continues until all the antitoxin is lost that is in excess of the amount that can be maintained by the new steady rate of production. Thus for a time, before the new steady level is reached, this excess antitoxin would be lost at the usual rate of disappearance of normal globulin. Holt (1950) claimed to have shown that the "pure" secondary response, or leap, shows a geometric loss in titre with time that corresponds well with one of the suggested rates of disappearance of globulin molecules. He calculated the "pure" secondary response by subtracting the primary base-line titre and the primary response at corresponding times from the total secondary response. In order to justify this procedure it is necessary to assume that the rest of the new antitoxin appearing after secondary stimulation is produced, both quantitatively and qualitatively, in a manner exactly similar to that produced by the same dose of antigen when it is employed as a primary stimulus. There appears to be no evidence that either of these assumptions is justified, and indeed it is known that the quality of antitoxin produced after primary stimulation is very poor, and that quality improves after successive injections of antigen (Jerne, 1951; Barr, 1951). It is unjustifiable to subtract titres of antitoxin of a certain quality from titres of another quality, for the apparent titres of non-avid antitoxin are dependent on the level at which titrations were

made. Holt's speculation is however of interest although the evidence given in support cannot be accepted.

Jerne (1951) commented on certain calculations made by us (Barr and Glenny, 1949) of the increase in antitoxic titre of a horse during the rapid rise of a secondary response; we estimated that during a certain period of 24 hours an average of one unit of antitoxin was produced every 30 seconds for every Lf of toxoid injected. Having shown that antitoxin produced in guinea-pigs after a second injection was of better avidity than antitoxin produced after only one injection, Jerne expressed the view that "a large part of the apparent increase in antitoxic titre after a second stimulus must be due to an increase in avidity of the antitoxin produced, and that the increase in output of antitoxic substance is probably much less dramatic." This comment may frequently be true when applied to the responses of animals to a *second injection*. It is unfortunate that certain workers now appear to regard the terms "secondary stimulus" and "second injection" as being interchangeable. It is difficult to understand how this idea has arisen, for it is well known that the first injection of antigen into a naturally immune animal, of unknown immunological history, may give rise to a secondary response. The secondary stimulus was originally defined by Glenny and Südmersen (1921) as "the term we apply to the injection of toxin into an actively immunised animal." In the 1949 paper we adhered to this definition, and the response in question was that to the fifth injection of diphtheria prophylactic given to this horse during a period of $2\frac{1}{2}$ years. At this stage, and indeed long before this injection was given, there was no evidence of non-avidity, and one of us (unpublished) has failed to obtain evidence of changes in avidity of antitoxin in successive samples taken during the sharp secondary rise in such horses. We are therefore of the opinion that the increases calculated by us (1949) cannot be accounted for by improvement in avidity during rapid production.

There is as yet no satisfactory explanation of the mechanism of antitoxin production. We believe that three separate activities may be involved. The first is the sensitisation of cells to respond to stimulation: this may be defined as the development of potential immunity, and can occur without the appearance of any measurable antitoxin in the circulation. The other activities are concerned with the appearance of antitoxin after specific stimulation, and with slow continued production which may be maintained for many years in the absence of further injection.

The effect of bleeding upon antitoxin production.

It has long been known that the withdrawal of large amounts of blood may have a stimulating effect on antibody production. Burnet (1941) says "there is general agreement that repeated bleedings do not reduce the antibody titre by the amount which would be calculated from the volume of plasma removed." In a review of the factors affecting the production of antibodies Cannon (1942) states that haemorrhage may "cause a reappearance in the blood of antibodies to previously injected antigens."

The experiments described here form part of a plan to utilise material collected in large-scale antitoxin production. They are examples of many observations on horses, giving similar results. Most of the work is concerned with diphtheria and tetanus antitoxins, both of which can be titrated with considerable accuracy.

Diphtheria antitoxin was titrated by the guinea-pig intracutaneous method, and tetanus antitoxin by the mouse subcutaneous method. Several tests were made at 10 per cent differences on all samples examined. Certain antitoxins such as tetanus and *Cl. oedematiens* α antitoxin, to which natural immunity is rare or absent, can be produced efficiently only if hyperimmunisation is postponed until several months after a few preliminary injections of the appropriate antigen have been given to establish basal immunity. In practice, horses with suitable natural immunity to other antigens such as diphtheria, staphylococcus or *Cl. welchii* α toxins, may be used during the resting period for the production of these antitoxins (Barr and Glenny, 1945). By following this procedure we have been able to utilise the testing period of many horses to immunise them against diphtheria, and to produce from them an average of 10 million units of diphtheria antitoxin per horse per month. By following the diphtheria antitoxin content of the serum of such horses during subsequent immunisation with other antigens, we have been able, in the course of routine production, to investigate factors influencing the rate of loss of actively produced antitoxin.

EXPERIMENTAL.

The Effect of Weekly Withdrawals of 8 litres of Blood.

Horse A received 1 course of immunisation with diphtheria toxoid and a further 7 courses with toxin plus alum: the average antitoxic titre reached in these 8 courses was 1070 units/ml., and that existing after the last course was 900 units. The horse was then used for the production of tetanus antitoxin, receiving 4 courses of injections and reaching an average value of 700 international units (1950). After each course of diphtheria injections and after the first 3 tetanus immunisations 3 bleedings were taken, each of 8 l. Four days after the last injection of tetanus toxin, 6 months after the last injection of diphtheria toxin, 8 l. blood were withdrawn, and the bleeding repeated 15 times at intervals of a week. Curve 1 in Fig. 1 shows the logarithms of the tetanus antitoxin titres and curve 2 those of the diphtheria antitoxin of successive bleedings. Each point after the first on both curves represents an antitoxic titre which is the resultant of a number of factors:

(*a*) the antitoxic titre 7 days earlier;
(*b*) loss due to destruction or elimination of globulin in the ordinary course of protein metabolism;
(*c*) the loss of antitoxin removed in the 8 l. blood withdrawn; this would amount to some 15 per cent of (*a*);
(*d*) the addition to the titre made by continued production; and
(*e*) the addition of antitoxin formed at the same time as normal globulin to replace that lost by bleeding.

It is reasonable to assume that the ratios of the amounts lost in *b* (routine loss) and *c* (removal by bleeding) in relation to *a* (original titre) would be the same for both antitoxins and represent the proportions of total globulin lost or withdrawn. Replacement rates depend upon the immunity state of the animal in relation to the two antitoxins. After the end of immunisation the rate of production rapidly declines but eventually reaches a steady level. Before this stage is reached loss under *b* (routine metabolic loss) is greater than gain under *d* (continued production),

and the titre would fall even without bleeding until it reached that level which could be maintained by continued production. Horse A should already have reached this stage in relation to diphtheria antitoxin, but curve 2 indicates a loss of 8·8 per cent per week for 3 weeks. The active production of tetanus antitoxin may have caused this loss, for evidence is given later in this paper that active production of one antibody in response to stimulation can reduce continued production of another. An alternative explanation is that continued production is not able to replace abnormal losses ; this may mean that the amount of antitoxin continually produced to replace routine wastage depends upon the number of modified globulin-producing cells, but that when excessive amounts of globulin are needed to replace abnormal wastage the normal cells are more active in production than some of those modified to produce antitoxin.

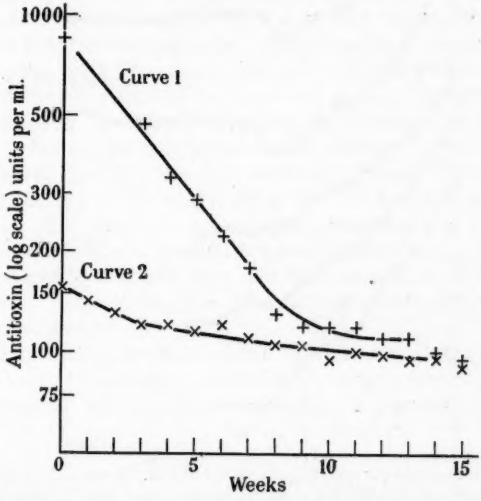

FIG. 1.—The decrease in tetanus (curve 1) and diphtheria (curve 2) antitoxin titres of weekly samples of serum from horse A from which 8 l. blood were withdrawn each week, commencing 6 months after the end of a series of diphtheria immunisations and 3 days after the end of tetanus immunisation.

The first 6 points of curve 1 lie near a straight line showing a steady exponential loss of 19·9 per cent per week. After the 8th week the rate of loss declines rapidly and becomes almost negligible. The increased loss after the 13th week can be accounted for by the fall in condition of the horse. If the graph of tetanus antitoxin for horse A is compared with a similar published curve for a horse on diphtheria immunisation which was not bled (Barr and Glenny, 1947), it will be seen that in the latter horse the change in slope was delayed until the 14th week. There was little difference between the proportions of antitoxin remaining in the two horses after 3 months ; that of the horse recorded in 1947 was 15 per cent and that of horse A was 12 per cent. This suggests that the effect of repeated withdrawals of blood was to lessen considerably the time when the antitoxin content was reduced to that which could be maintained by continued production.

It is difficult to understand the abruptness of the change in the rate of loss ; it cannot be due to adjustment to repeated removals of blood, because the same abrupt change occurs in horses from which large amounts of blood have not been removed.

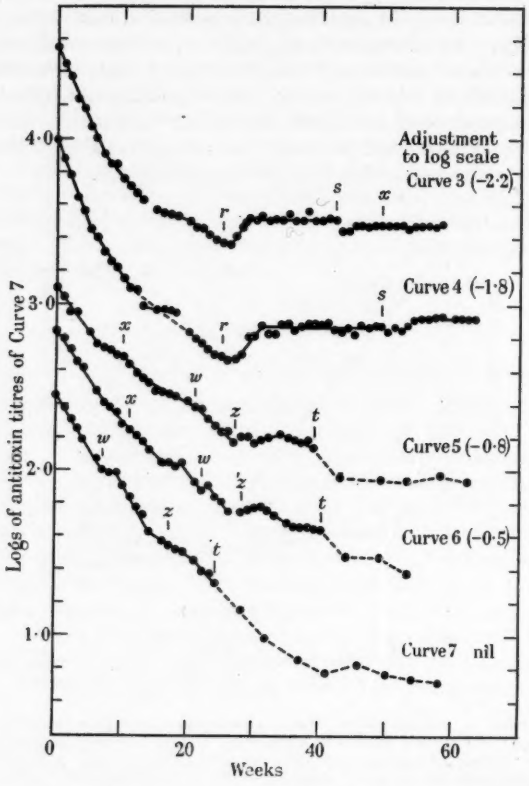

FIG. 2.—The decrease in diphtheria antitoxin titre of horse B (curve 3) and horse C (curve 4) bled 8 l. each week for 26 weeks, compared with control horses D, E and F (curves 5, 6 and 7) which were not bled until the end of other immunisations. Adjustments are made to the logarithmic scale to enable the five curves to be included in one figure.

Treatment of horses.

r. Last weekly bleeding.
s. Single bleeding of 8 l.
t. First of 3 bleedings, each of 8 l., taken within a period of 8 or 9 days.
w. First of a series of 5 injections, within 15 days, of *Cl. welchii* B or D filtrate.
x. Start of main course of scarlet fever toxin.
z. Start of main course of injections of *Cl. welchii* B or D filtrate.

Curve 2 is very different from curve 1 because the diphtheria antitoxin had been produced by immunisation which was terminated 6 months before the beginning of this experiment, and continued production was enough to balance metabolic loss. The chief interest in the two curves lies in the evidence that the titres

of different antitoxins may fall in the same horse at different rates during the same time. The titres of the two antitoxins at the end of the experiment were 11·2 per cent of the tetanus antitoxin present 15 weeks earlier and 10·0 per cent of the diphtheria antitoxin present at the end of immunisation 9 months before.

In a further experiment 5 horses were given a short course of injections of diphtheria toxoid, and 8 l. blood were then withdrawn on 3 occasions within 8 days. Observations of antitoxic titres were begun 7 days after the last bleeding, when horses B and C were again bled 8 l., this procedure being repeated each week for a further 25 times. Diphtheria antitoxin was titrated in samples of serum from these horses (curves 3 and 4 in Fig. 2) and from the others, D, E and F (curves 5, 6 and 7), which acted as controls without bleeding. Most of the points representing the logarithms of antitoxic titres tend to fall on smooth curves of a shape suggesting the resultant of exponential loss partially balanced by continued production; this eventually maintained a constant level of antitoxin after the amounts present had fallen considerably. Superimposed on these smooth curves are minor fluctuations, with the result that most of the points fall on a series of straight lines. The first portions of curves 3 and 4, continuing as far as the points marked *r*, at which time the last weekly bleedings were taken, and the parts of curves 5, 6 and 7 as far as the points marked *t*, comprise a total of 145 points, of which 121 are covered by straight line sections: these extend over at least 4 complete weeks (5 points). Of these points, 92 (*i.e.*, 76·0 per cent) are within 2·5 per cent of the line, and 111 (91·7 per cent) are within 5 per cent. These linear sections suggest that there is a general tendency for actively acquired antitoxin to be lost at a constant logarithmic rate which is subject to slight change through alterations in normal metabolism or in the rate of continued production of antitoxin.

In Table I a comparison is made of the rates at which antitoxin was lost by the five horses. It will be seen that in the early stages horses B and C lost antitoxin at almost the same rate that tetanus antitoxin was lost by horse A (Fig. 1).

TABLE I.—*The Rates of Loss of Diphtheria Antitoxin in the Serum of Horses B and C, from which 8 l. Blood were Withdrawn each Week for 26 Weeks, Compared with the Rates of Loss in Control Horses D, E and F.*

Period in weeks.	Percentage weekly loss of antitoxin.					Average weekly loss.		
	B.	C.	D.	E.	F.	B and C.	D, E, F.	D and E.
0 to 7	19·3	20·1	11·1	13·5	13·9	19·7	12·8	—
7 to 24	6·8	8·0	6·2	7·4	8·8	7·4	7·5	—
7 to 31	3·5	4·1	5·1	5·8	—	3·8	—	5·4
0 to 31	7·4	8·0	6·5	7·5	—	7·7	—	7·0
0 to 39	6·0	6·3	5·6	6·8	—	6·2	—	6·2

During the first 7 weeks the average amount of antitoxin lost by B and C was 6·9 per cent more than the average amount lost by the control horses: thus it would appear that at least half the antitoxin removed by bleeding was replaced every week. During the next 17 weeks the average rates of loss were the same for both groups, but the three horses that did not have weekly bleedings can be regarded as strict controls only for the first 7 to 10 or 11 weeks. Horses D and E had unsuccessful courses of injections of scarlet fever toxin lasting from the 10th to the

20th week and from the 11th to the 21st week respectively. Starting one week after the end of the course, both horses received 5 injections of *Cl. welchii* type B filtrate, doses being given twice a week. Six weeks later, during which interval the horses were rested, the main course of immunisation with type B filtrate was begun. Horse F received the preliminary injections of this filtrate starting at the 7th week of the experiment, and the main course was begun 11 weeks later. The effect of these injections of other antigens on the rate of loss of diphtheria antitoxin will be discussed later.

It will be seen that curves 3 and 4 are roughly parallel from the 2nd to the 18th week, apart from minor fluctuations. During this period the antitoxic titre of horse B averaged 1·59 times that of C, and on 14 of the 16 occasions the ratio of the titres was within 5 per cent of this figure. The titres of B and C were approaching their final level and the effective loss (due to the excess of loss from metabolism and bleeding combined over continued production) was greatly reduced and, as shown in Table I, approached that of the control horses. There is also uniformity in the general shape of curve 3 with that of 5 between the 10th and the 22nd week and with that of 6 between the 8th and the 18th week. This uniformity suggests that the titres of horses D and E were approaching a constant level towards the end of the periods named. These titres were approximately double those of B and C measured in terms of the original titres; the levels were not, however, maintained and, as shown in Table II, eventually fell to levels closely corresponding to those of the horses which had been repeatedly bled. The reasons for this will be discussed later.

TABLE II.—*The Amount of Diphtheria Antitoxin Remaining after Different Intervals Expressed as a Percentage of the Amount Present at the Start of the Experiment.*

Time in weeks.	Per cent amount of antitoxin remaining.					Average amount remaining.		
	B.	C.	D.	E.	F.	B and C.	D, E and F.	D and E.
7	22·2	20·7	44·0	36·3	35·2	21·5	38·3	—
24	6·7	5·0	14·7	9·9	7·3	5·8	10·2	—
26	6·3	4·6	13·8	8·3	—	5·4	—	10·7
31	9·4	7·7	12·4	8·8	—	8·4	—	10·4
39	8·8	7·7	10·8	6·3	—	8·2	—	8·2

The antitoxic titres of horses B and C were tested on samples of serum taken daily after the bleedings at the 15th week and those of C during the following week as well. The same general curve was obtained in all these cases and the average titres are shown on curve 8 in Fig. 3, where the points represent the percentage of the amount present at the beginning of each week. The lowest value is reached on the 2nd day after bleeding and remains at about 88 per cent for a further two days and then shows a rapid rise during the next 2 days, reaching an average of 94·5 per cent of that present at the time when 8 l. were bled. Around this time the average weekly loss was 3·0 per cent from horse B and 2·3 per cent from C, but at the 18th or 20th week the rate of loss increased appreciably. There is considerable evidence that this increased loss was due to the inability of the horses to replace globulin any longer. The last weekly 8-l. bleeding (*r*) was taken at the 25th week and the titre of both horses began to rise after the 26th week,

that of B increasing by 44·8 per cent and that of C by 40·8 per cent in 3 weeks. Table III shows that the increase in titre corresponded to an increase in protein. Curve 3 (Fig. 2) shows that a constant titre was reached by B 4 weeks after the last weekly bleeding, and that the antitoxic level reached corresponded to that

TABLE III.—*The Protein and Antitoxin Content of Serum Samples taken from Horses B and C, 2 and 5 Weeks after the last of 26 Weekly Bleedings of 8 l., and Similar Figures for Control Horses D and E.*

		Horse.			
		B.	C.	D.	E.
Protein (g./100 ml.)	2 weeks	5·53	4·97	7·89	7·40
	5 „	7·14	6·85	8·00	7·37
Antitoxin units/g. protein	2 weeks	290	146	296	297
	5 „	282	143	290	323

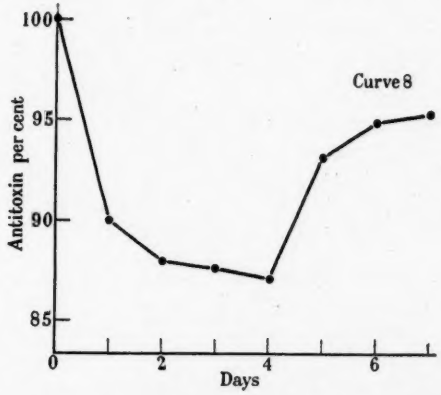

FIG. 3.—The decrease and subsequent increase in the diphtheria antitoxin titre after the removal of 8 l. blood. The figures given are averages of titres at and subsequent to the 16th weekly bleeding of horse B and the 16th and 17th of C.

on the 20th week. The titre of C fluctuated considerably before a steady level was reached, and this was above that found at the 20th week.

A further 8-l. bleeding was taken from B at the 42nd week (*s* in Fig. 2). The antitoxin titre fell by 13·9 per cent, and from the 45th week onward a steady titre was maintained at a level 7·5 per cent lower than that maintained for 13 weeks before this bleeding. The further 8-l. bleeding from C at the 49th week was followed by a loss of 7·9 per cent, and after 4 weeks a new constant level was reached 15·9 per cent above that maintained for 15 weeks before this bleeding. No explanation is offered for these changes in either direction.

All five horses were used for varying lengths of time for the production of other antitoxic serum. Titrations for diphtheria antitoxin content were made on weekly samples from these and a number of other horses, including G and H which had had courses of immunisation with diphtheria toxin before being transferred to other productions, and J and K which were purchased as young foals and given periodic injections of diphtheria toxoid or A.P.T. spread over 3 years. The great

reduction during recent years in the number of horses with natural immunity to diphtheria had necessitated this type of preparation. These two horses however were not eventually needed to produce diphtheria antitoxin and were used for other purposes. Our general procedure for the hyperimmunisation of horses, after basal immunity has been fully established, has been to give a course of intramuscular injections of toxoid or toxin until titrations on frequent, usually weekly trial bleedings show that the horse has reached a stage when it can profitably be bled. Three 8 l. bleedings are then taken within a period of 8–10 days. After the last of these bleedings the horse is rested for 10 days or more and then given a rapid course of 5 injections of toxin with alum on alternate days. A few days after the last injection the 1st of another series of 3 bleedings is taken. If the condition of the horse allows, this rapid course is repeated every 4 weeks but longer rests are frequently given.

In the work recorded in this paper test bleedings were taken every Monday irrespective of any other treatment that the horse was undergoing. After the end of the preliminary course of immunisation or each rapid course the 1st bleeding of 8 l. was taken almost invariably on Mondays or Tuesdays, followed by a second bleeding 3 days later and the 3rd bleeding 7 to 9 days after the first. Thus for each series of 3 bleedings the antitoxic titre at the time or just before the 1st bleeding was known. Between this observation and the next one, however, the horse would have been bled 8 l. on two separate occasions, and the 3rd bleeding would be taken at the time of this next observation or on the following day. The various curves show that the diphtheria antitoxic content frequently falls during immunisation with other toxins but a new lower general level is soon reached.

For a general level of antitoxin to be maintained the loss due to these 3 bleedings must be made good. Table IV shows the course of fall and then rise in titre during each immunisation starting at or immediately before the first bleedings. The observations are limited to consecutive immunisations starting at the earliest time at which a general level of diphtheria antitoxin was maintained. The figures

TABLE IV.—*The Average Percentage Fall and Subsequent Rise in the Residual Diphtheria Antitoxic Titres after the 1st of a Series of 8 l. Bleedings.*

	Horse.						
	J.	K.	F.	B.	G.	H.	All.
Number of consecutive heterologous immunisations	8	4	8	11	5	7	(80)
Blood previously withdrawn (l.)	0	68	120	368	440	728	
Period of previous observation (months)	40	43	22	22	37	38	
Number of injections of diphtheria antigen	6	8	14	15	62	72	
Interval since last such injection (months)	10	13	10	19	12	10	
Approximate titre in units/ml.	1·7	4·5	5	9	45	16	
Interval since first of the 3 bleedings (weeks)	Mean percent. remaining on successive weeks after the 1st of 3 bleedings each of 8 l. taken within a period of 8–10 days						
1	84·1	84·3	84·2	87·6	87·2	81·0	85·9
2	83·1	81·9	84·3	82·9	79·4	82·7	83·5
3	92·8	85·1	89·7	86·7	85·1	88·6	88·7
4	97·2	94·8	94·0	94·5	95·3	96·4	96·5
5	101·7	—	103·6	99·7	99·1	101·6	99·7
6	—	—	102·3	105·5	—	111·1	104·8

given for each horse are calculated from the geometric means of the antitoxin titres at corresponding times in weeks after the start of the series of 3 bleedings, and given as a percentage of the geometric means of the titres at the start of each series. There is close agreement in the loss and subsequent gain in antitoxin for each horse, although the histories of the horses were so varied. The last column gives the average of 80 series limited to the same 6 horses, but taken on all occasions when the fall in titre was followed by an equivalent rise. As a general rule 4 weeks after a set of 3 bleedings was not a long enough period for recovery to be complete. The average titre after 5 weeks was about the same as that at the start and at 6 weeks it was higher. The importance of this Table lies in the evidence it gives that recovery after withdrawal of blood is not influenced by previous experience of bleeding. Horse J had never been bled on any large scale during the 3 years it was under observation until the 1st of the series of bleedings recorded in this Table; horse H had had 8 l. blood withdrawn on 91 separate occasions. This evidence against any effect of previous experience of bleeding suggests that the reduced rate of loss from horses B and C after the 7th week of bleeding was not due to experience, but probably caused by the reduced titre approaching that which could be replaced by continued production.

Effect of Other Immunisations on Residual Diphtheria Antitoxin Content.

It is well established that "crowding out" of antibody production may occur (*a*) if an antigen is injected at a time when active production of another antibody is occurring, and (*b*) if two antigens are injected at the same time and one of them is in excess.

The earliest work on this subject concerned interference due to precipitin formation, with the responses of animals to the injection of other antigens. Benjamin and Witzinger (1912) showed that a preliminary injection of horse serum inhibited the formation of sheep cell haemolysin in rabbits. Lewis (1915) found that an amount of protein that would produce marked anaphylactic sensitisation when injected alone into a guinea-pig, failed to do so if it were injected together with, or 24 hr. after a much larger amount of another protein.

Glenny, Hopkins and Waddington (1925) showed that an injection of diphtheria toxin-antitoxin mixture or of toxoid into immune rabbits incited little or no antitoxic response if given at a time when the rabbits were producing precipitin to horse, goat or cow serum. Such active precipitin formation interfered also with the primary response to diphtheria toxoid. Glenny and Waddington (1928) injected guinea-pigs with diphtheria toxin-antitoxin mixtures, either alone or before, at the same time or after an injection of normal horse serum. No interference occurred when the serum was given 4 weeks earlier or 7 days later, but when it was given one or two weeks before, or at the same time as the toxin-antitoxin mixture, there was a decreased diphtheria response. Similar results were obtained when a single dose of diphtheria toxoid was given in place of the toxin-antitoxin mixture: interference with the diphtheria response occurred when horse serum was given at the same time or 2, 4 or 6 days before the injection of toxoid. The same authors, using a combined diphtheria and scarlet fever prophylactic, showed that an excess of the scarlet fever toxin depressed the response of guinea-pigs to the diphtheria component.

It appears possible from these results that crowding out would occur also if

an animal which had good potential immunity to one antigen and little or none to another antigen were injected simultaneously with both. Examples of alternate preferential response to one component of filtrates containing multiple toxins are to be found in the work of Glenny, Barr, Llewellyn-Jones, Dalling and Ross (1933). They published curves showing the course of production of *Cl. welchii* α and β antitoxin in horses injected with *Cl. welchii* type B filtrates containing α and β toxins. One of these horses had some naturally produced β antitoxin present before injection, but had no detectable α antitoxin and apparently no potential immunity to α toxin. During the first 4 weeks of immunisation the β titre therefore rose rapidly but no α antitoxin was produced. During the 5th and 6th weeks however, after basal immunity had been established, the α titre rose rapidly and the β value showed a considerable fall. After the 6th week the β value once again rose rapidly and the α titre fell slightly. Another horse with some normal α but no normal β antitoxin produced α before β, but when the β antitoxin was rising to its maximum value the α titre fell.

In the work recorded in this paper we have had an opportunity to study the effect of immunisation with other antigens on the continued production of diphtheria antitoxin in horses. We shall refer to this immunisation with other antigens as "heterologous immunisation." Horses D, E and F were all used to prepare lamb dysentery antitoxic serum. The diphtheria titres of F shown in curve 7 fell more than those of D and E (curves 5 and 6); this horse was very successfully immunised with *Cl. welchii* type B filtrate; D and E after unsuccessful courses of injections with scarlet fever toxin received type B filtrates but neither responded so well. The scarlet fever immunisation had little effect upon the slope of curves 5 and 6, but it will be noted that during preliminary injections of *Cl. welchii* type B filtrates, at the time marked *w*, the diphtheria titres were almost stationary for one or two weeks and then fell more rapidly than they had done before these injections. The first main immunisation starting at *z* had a bigger effect upon F than upon the other two horses. At the point marked *t* the first 8 l. bleeding was taken, followed by two further bleedings in the next 7 or 8 days. Starting about 10 days later 5 further injections were given on alternate days and a series of 3 bleedings again taken. These rapid courses were repeated every 4, 5 or 6 weeks. As a result of these bleedings the diphtheria titres fell considerably for 2 weeks and then increased and sometimes reached their former level in about 5 weeks. To avoid making the shape of the curves too complicated the intermediate points have been omitted in Fig. 2, and the only titres recorded are those at the time of the 1st bleeding of each immunisation. The general shape of the intermediate portions of the curves is shown later in Fig. 6.

The diphtheria titre of each horse fell by 25 to 30 per cent from the end of the first immunisation to the end of the second. The titre of F continued to fall during 3 further immunisations, that of D remained almost stationary during 4 subsequent immunisations while that of E did not fall between the 2nd and 3rd immunisations but did during the following one; this horse was then in poor condition and was discarded. Table V gives comparative figures for the amount of diphtheria antitoxin in these horses and in B and C one year after the start of the experiment. It is suggested that the production of high grade *Cl. welchii* β antitoxin had considerable effect in reducing the residual diphtheria antitoxin content of horse F. The *Cl. welchii* β units quoted are provisional units which have been in use at the Wellcome Research Laboratories for over 20 years;

TABLE V.—*The Effect of Immunisation of Horses with* Cl. welchii *Type B Filtrate upon their Diphtheria Antitoxin Contents.*

Horse.	Diphtheria antitoxin per cent remaining after one year.	Number of 8 l. bleedings.	Immunisations started.	*Cl. welchii* 1st imm.	β titres, average, 2nd and 3rd.
B	8·3	27	None	—	—
C	7·3	27	,,	—	—
D	6·9	9	10th week	800	1800
E	4·3	9	11th ,,	1500	2200
F	2·4	21	7th ,,	2700	6370

the *Cl. welchii* ε unit referred to later in this paper was fixed shortly after the descriptions of the various toxins were published by Glenny *et al.* (1933). It greatly

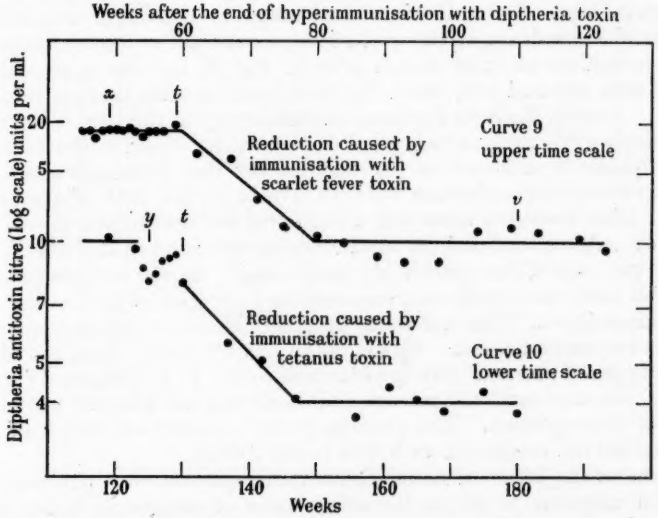

FIG. 4.—The effect of immunisation with scarlet fever toxin (curve 9) and later with tetanus toxin (curve 10) on the diphtheria antitoxin content of horse B.

Treatment of horse.

t. First of 3 bleedings, each of 8 l., taken within a period of 8 or 9 days.
v. Single injection of 10 ml. tetanus A.P.T.
x. Start of main course of scarlet fever toxin.
y. Start of main course of injections of tetanus toxin.

facilitates work on the production of new antitoxins if provisional units are fixed as early as possible, and it is always hoped that subsequent workers will make use of the same unit.

Further evidence of the influence of heterologous immunisation on continued production is given in Fig. 4. Curve 9 shows the diphtheria antitoxin titres of horse B: it is a continuation, with some overlap, of curve 3 (Fig. 2), and curve 9 itself is continued in curve 10. The time scale for curve 9 is at the top of the diagram and that for curve 10 at the bottom. The previous history of the horse can be summarised thus. Antitoxin titre one week after the third bleeding following a short immunisation with diphtheria toxoid was 225 units/ml. After

one year during which 26 weekly bleedings of 8 l. were taken the titre had fallen to a steady level of 18·9 units/ml., *i.e.*, 8·4 per cent of the original titre : curve 9 starts at this point. From weeks 45 to 52, 8 weekly test samples each about 20 ml. of serum all contained within 5 per cent of this amount of antitoxin. Scarlet fever immunisation was started at week 49 (*x*) : the first immunisation did not depress the diphtheria antitoxin titre by more than 5 per cent, but subsequent short courses of immunisation each followed by 3 bleedings of 8 l. decreased the titre at first rapidly and then more slowly until week 93. When the interval between courses of immunisation was increased to 6 weeks the titre increased slightly. The mean level of antitoxin from week 80 to 123 was 9·9 units/ml., with a maximum discrepancy of 10·2 per cent.

Tetanus immunisation started at week 125 (*y*), and the first full bleeding was taken 5 weeks later. Curve 10 shows the changes in diphtheria titre following the last group of 3 bleedings at the end of the scarlet fever immunisation. These changes are similar to those shown later in Fig. 6, but the titre suddenly fell between weeks 129 and 130, when the first bleeding after tetanus immunisation was taken. During the next 3 courses of immunisation the titre fell considerably but once again a relatively steady level was reached. From weeks 147 to 180 the titres of diphtheria antitoxin at the time of the first bleedings after each of 7 courses of tetanus immunisation were all within 10 per cent of a mean of 3·89 units/ml. After week 180 titres fell as the horse lost condition ; it was discarded at week 188. After scarlet fever immunisation the level of diphtheria antitoxin fell to 52·4 per cent of that previously maintained ; during tetanus immunisation the level fell more, and at the end was only 39·3 per cent of that at the beginning of this immunisation. This difference may indicate more immunological activity during tetanus immunisation ; this is borne out by the average protein content of the two types of plasma. We are indebted to Dr. F. V. Lingood for the information that tetanus antitoxic plasma almost always has a higher protein content than scarlet fever plasma. The average protein content of 1000 l. of the latter was 7·03 g./100 ml. compared with 8·04 in the former.

Fig 5 shows the effect of heterologous immunisation on diphtheria antitoxin produced in response to prophylactic injections of antigen in horses which had not received courses of hyperimmunisation with diphtheria toxin or toxoid. Horse J (curve 11) received its first injection of diphtheria A.P.T. when 12 months old ; several further injections were given, sometimes accompanied by tetanus A.P.T., during the next 2 years. Twenty-nine months after the 1st injection a 6th dose of diphtheria A.P.T. (5 ml.) was given. A typical secondary response occurred ; the highest titre of horse J was 16·4 units/ml. reached 10 days after injection ; the subsequent fall in titre was typical, and a level was reached 13 weeks after injection. From week 13 to week 22, 10 weekly samples of serum all contained within 2 per cent of 4·86 units/ml., 29·6 per cent of the highest titre. Although it is usual for the curves of antitoxin content to flatten out in this manner, such close consistency in the successive titres is unusual. During weeks 19 to 22 this horse received preliminary injections of *Cl. welchii* type D filtrates (*w*), causing titres to fall to a new level reached at week 30 ; during the succeeding 8 weeks 6 samples of serum contained within 3·1 per cent of a mean titre of 3·26 units, 67·1 per cent of the former level. Two samples at weeks 34 and 35 contained 11·2 and 4·7 per cent above this value. The main course of immunisation (*z*) had started at week 31 ; a cautious dosage is usually adopted and the first few

injections may have little effect, but in the course of this work it has almost invariably been found that a slight rise in diphtheria titre occurs shortly after heterologous stimulation, followed by a fall when rapid production of the new antibody occurs. Between weeks 37 and 44 the titre fell to 2·08 units/ml. when the first bleeding was taken ; a new level was reached shortly afterwards, and from week 52 to 77, 5 of 6 bleedings at the end of subsequent immunisations were within 3·4 per cent of 1·89, 58·0 per cent of the previous level. This titre was not however maintained, and during the next 7 months it fell gradually to 1·38 unit/ml. Horse J was a very successful producer of *Cl. welchii* ε antitoxin, its titres reaching 800 provisional units/ml.

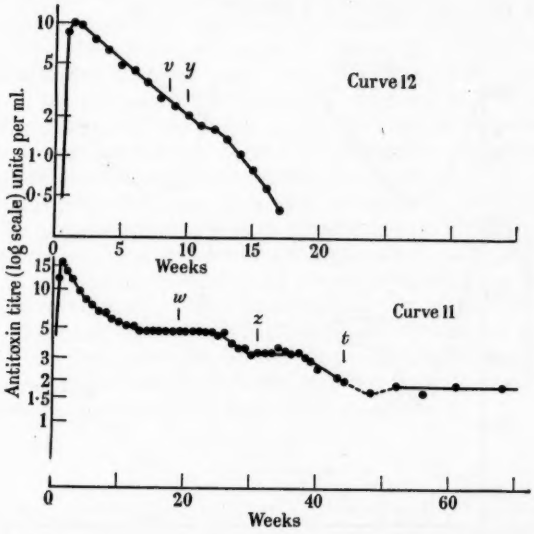

FIG. 5.—The effect of heterologous immunisation upon the diphtheria antitoxin titres produced in response to prophylactic injections only. Curve 11 shows the response of horse J to its 6th injection, curve 12 that of horse L to its 2nd injection of diphtheria prophylactic. This horse was naturally immune before the 1st injection was given.

Treatment of horses.

t. First of 3 bleedings, each of 8 l., taken within a period of 8 or 9 days.
v. Single injection of 10 ml. tetanus A.P.T.
w. First of a series of 5 injections, within 15 days, of *Cl. welchii* B or D filtrate.
y. Start of main course of injections of tetanus toxin.
z. Start of main course of injections of *Cl. welchii* B or D filtrate.

The last horse of this series, L (see curve 12), was naturally immune to diphtheria (normal value 0·5 unit/ml.) and received an injection of 10 ml. A.P.T. 6 months before the beginning of this experiment. At the time of a second injection of A.P.T. the diphtheria antitoxic titre was 0·31 unit/ml., 7 days later it was 8·5, at 10 days 10·0 and at 14 days 9·5 units/ml. After this time the titre fell exponentially at the rate of 17·1 per cent per week. Sixty days after the diphtheria injection the horse received an injection of 5 ml. tetanus A.P.T. (*v*), its 4th injection of

this antigen, and 10 days later the main course of immunisation with tetanus toxin (*y*) was begun. The diphtheria titre fell very little between weeks 11 and 13; this may have been due to the heterologous injections, but on the other hand the curve of antitoxin content of most horses tends to flatten out about this time after injection. By the 13th week however the titre had again fallen, and during the next 4 weeks the rate of loss averaged 26·8 per cent per week. The rate of loss of antitoxin from any animal does not increase in this manner unless the animal is producing other types of antibody or is falling away in general condition. Indeed a considerable reduction in the rate could have been expected by this time. The antitoxic titres of horse L at weeks 16 and 17 were respectively 20 and 29 per cent lower than they would have been if the rate of loss from the 2nd to the 11th week had continued for a further 6 weeks.

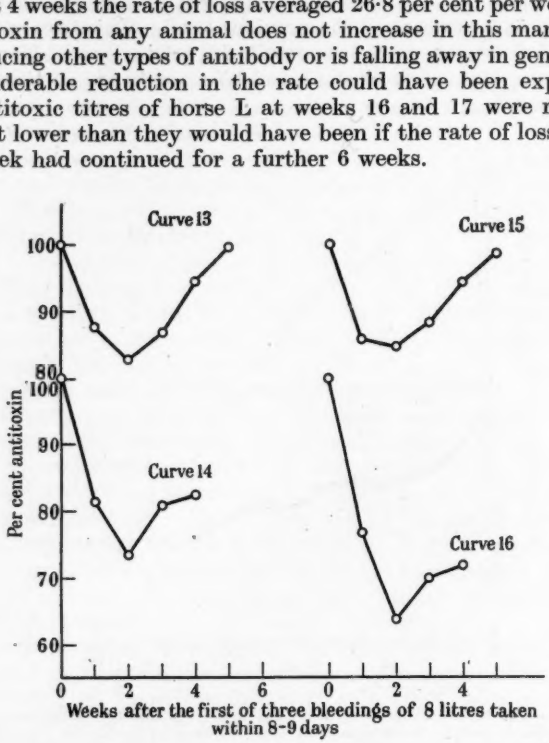

FIG. 6.—The decrease and subsequent increase in diphtheria antitoxin titre after the 1st of 3 bleedings of 8 l. taken at the end of a course of immunisation. Curves 13 and 15 show the average results after immunisation of horses B and F at a time when their general level of continued production was maintained; curves 14 and 16 from the same horses when the level was falling.

It has been seen that heterologous immunisation reduces the level of diphtheria antitoxin that can be maintained by continued production. The reduction to the new level can take place without large bleedings being taken from the horses as in curves 11 and 12; the reduction can continue during several short immunisations followed by bleedings as in curves 7 and 10; or the reduction may not be apparent until the first 8-l. bleedings are taken as in curve 9. In every instance a new level of antitoxin is reached and maintained for many weeks, during which further immunisations are given and bleedings taken. The rates of loss and recovery have been shown in Table IV, and in Fig. 6 a comparison is made of the

change in antitoxin content after bleeding during the two different phases (*a*) when the level has been maintained over a long period and (*b*) when further loss was still occurring. Curve 13 has been drawn from the figures given in Table IV for 11 immunisations of horse B ; curve 14 shows the average of 8 immunisations when the level was falling. In the first two weeks the 3 bleedings of 8 l. have lowered the initial titre by 17·1 per cent in curve 13 and by 26·6 per cent in curve 14. On the other hand the respective increases from the 2nd to the 4th week differed only as 11·6 to 8·9. Curve 15 shows the average figures for 19 immunisations of horse F when the level was maintained, and curve 16 for 3 immunisations when the diphtheria titres were falling. The figures corresponding to those just given are losses of 15·4 and 36·3 per cent during the first 2 weeks and increases of 9·6 and 8·1 per cent during the next 2 weeks. They show that during a period of a number of successive immunisations and corresponding bleedings the loss due to bleeding alone is almost made up before the next bleedings occur, but in addition to loss due to bleeding there is a further loss which does not occur when the general level of continued production is maintained. It is reasonable to suppose that this additional loss is due to interference with continued production caused by the active production of the heterologous antibody. After several heterologous immunisations this interference no longer occurs.

We are now investigating one other way in which heterologous immunisation and possibly bleeding may interfere with production of diphtheria antitoxin after secondary stimulation. An injection of diphtheria prophylactic, if given at certain times in relation to injections of other antigens, may give a very weak and sometimes slow response. One example may be given here : a horse with a history similar to that of J produced 63, 70 and 67 units/ml. after the 4th, 5th and 7th injections. The 6th injection of diphtheria prophylactic given at a time of strong heterologous interference produced only 3·4 units/ml. and the height of the response was not reached until 71 days after injection.

DISCUSSION.

The main object of this paper is the provision of additional facts concerning the production and loss of antitoxin. These facts may assist eventually in formulating a rational theory of antitoxin production : at the present time the grounds for speculation are not very firm.

The rapid production and subsequent rapid loss of antitoxin that occur after secondary stimulation or after hyperimmunisation suggest a sudden increase in the number of antitoxin-producing cells, followed by an even more rapid cessation of activity of these cells caused by either a great reduction in their number or by a loss of their specialised function. If production of antitoxin entirely ceased and there was no readjustment of total protein content or of relative amounts of globulin to other proteins, antitoxic titres would decrease exponentially. In any animal globulin is being constantly lost and replaced ; there is every reason to believe that antitoxic globulin is lost at the same rate as normal globulin, and would be replaced by normal globulin if production of a particular antitoxin ceased. It follows that the amount of this antitoxin lost at any moment of time would be directly proportional to the amount of this particular antitoxic globulin present in relation to all other globulins. It follows that the logarithms of the particular antitoxic titres plotted against time fall upon a straight line.

The situation is, however, more complicated than this. Production does not cease entirely and some of the antitoxic globulin lost is replaced. While the total amount of globulin replaced balances the amount lost, the proportion of the newly formed globulin that is antitoxic depends upon the number of antitoxin-producing cells relative to the total number of globulin-producing cells. The reduction in numbers of antitoxin-producing cells after the height of antitoxin production appears to be very rapid, but how quickly the new level of population is reached is not known. As the titres fall, so the amount lost each week falls until the rate of production is equal to the rate of loss. After this takes place the titre will remain constant unless the total globulin content alters or the relative number of antitoxin-producing cells changes. Withdrawal of blood reduces the total protein content and the antitoxin content falls to the same extent. If this happens when the antitoxin content is above that which can be maintained by the population of sensitised cells the fall in titre reduces the time before destruction and replacement are balanced. If this state has already been reached, the protein removed by bleeding has the same proportion of antitoxic globulin as the protein freshly formed to replace loss, and the antitoxic titre of the blood will again reach the level maintained before bleeding.

One other effect must be added to the simple idea of loss (dependent on content) and gain (dependent on number of available producing cells). Accompanying the rapid production of antitoxin there is an increase in globulin content. When rapid production of antitoxin ceases the rate of production of fresh protein may be temporarily reduced or the rate of destruction or elimination increased until the total protein content falls to its normal level.

It is difficult to assess the extent to which non-specific factors affect active production of antitoxin, because it is not possible always to judge what would have occurred in their absence. Interference with continued production is, however, easier to assess. The injection of another antigen often appears to increase temporarily the continued production of a former antibody. This may be due to a temporary increase in production of serum globulins. When, however, active production of another antibody is established, there is a fall in the formerly constant antitoxin level probably due to a reduction in the proportion of special cells. The rapid increase in other specialised cells causes some form of " crowding out," and the former proportion of cells engaged in constant production of the original antitoxin is permanently reduced. Thus the position again arises of routine elimination of protein removing antitoxin faster than it can be reproduced. This continues until a fresh antitoxin level is reached when loss and production are balanced. This new level may be maintained for many weeks, even though the animal may be maintaining also a high titre of the newly produced heterologous antibody. At this stage, however, the amount of *rapid* production of the heterologous antibody may be fairly small if the titres do not increase with successive immunisations or fall much during resting periods.

There is already definite evidence that the injection of an antigen at the time of active production of heterologous antibody may greatly reduce the response to this antigen. This fact and our observations on continued production should be used as a warning against the indiscriminate use of combined prophylactics, and the injection of one type of prophylactic following injection of another type without reference to the existing immunity states of the individuals concerned.

SUMMARY.

After secondary stimulation or after hyperimmunisation antitoxic titres increase rapidly and then decrease until a relatively permanent antitoxic titre is reached some months later.

After the peak of production the greatly increased number of antitoxin-producing cells rapidly falls to a new level capable of maintaining a constant level of production.

The turnover of globulin maintains a constant level of total globulin, but the amount lost at first has a higher content of antitoxin than has that produced for replacement.

Removal of blood acts in the same way as routine turnover; it results in additional loss of antitoxin if the blood removed has a higher antitoxin content than can be replaced, but if a constant level of antitoxic titre is being maintained antitoxic and normal globulin are replaced in the same proportion as was removed.

Antitoxic response to another antigen reduces the number of cells modified by the former antigen and the permanent level of antitoxic titre is reduced.

Secondary stimulation with one antigen evokes little response if given during the time of active response to another antigen. In the light of this finding prophylaxis with combined antigens needs careful investigation.

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THE INFLUENCE OF CORTISONE ON THE REGENERATION OF TENDON AND TRACHEAL EPITHELIUM IN THE RAT.

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THE effect of cortisone on tissue growth and regeneration has recently been extensively studied. In connective-tissue production the influence of cortisone has been found to vary according to the circumstances of the experiment, *i.e.*, the species of animal, the means employed to produce the new connective tissue, and the dosage and method of administration of the drug. Ragan, Howes, Plotz, Meyer and Blunt (1949) and Bangham (1951) observed no dramatic effect of cortisone on fibrous tissue growth in healing wounds of the skin of rats. On the other hand, Coste, Basset and Delaunay (1951) found that in response to subcutaneous injections of turpentine in cortisone-treated rats there was a reduced number of fibroblasts and almost no production of new vessels or fibres, whereas in the control group of animals vascular connective tissue developed in response to this irritation. Magarey and Gough (1952) demonstrated a retardation of fibrous tissue development around powdered quartz introduced into the peritoneal cavity of rats which were receiving 5 mg. cortisone daily.

Published reports on the effect of cortisone on epithelial regeneration deal only with squamous epithelium, and are conflicting. It was found by Creditor, Bevans, Mundy and Ragan (1950) in man, by Ragan, Howes, Plotz, Meyer, Blunt and Lattes (1950) in rabbits, by Bangham (1951) in rats and guinea-pigs, and by Baxter, Schiller and Whiteside (1951) in man that cortisone exerts no appreciable influence on the rate of regeneration of epithelium in skin wounds. Ashton and Cook (1951) also found no effect on the growth of corneal epithelium in rabbits, using small doses of cortisone. On the other hand, Howes, Plotz, Blunt and Ragan (1950), Baxter, Schiller, Whiteside and Straith (1951) and Bangham (1951) found that epithelial repair was delayed in skin wounds of rabbits. Leopold, Purnell, Cannon, Steinmetz and McDonald (1951) and Ashton and Cook (1951) considered that regeneration of corneal epithelium in rabbits was reduced by cortisone given in large doses.

This paper deals with the effect of cortisone on the repair of connective tissue and epithelium. Regenerating tendon provides excellent material for the study of fibrous tissue formation, for the newly-formed band of tissue can easily be identified. Moreover, if tenotomy is carried out aseptically, the process can be studied without the complication of the severe inflammatory reaction encountered in open skin wounds. In choosing the trachea for the study of epithelial regeneration one approaches, as nearly as possible, a study of epithelial growth *per se*,

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i.e., epithelial growth relatively uncomplicated by the underlying granulation tissue. With the technique which we have used the regenerating epithelium progresses over a ready-made surface, *viz.*, the elastic layer of the lamina propria. The possibility of any effect on epithelial growth being primarily dependent on an effect on the granulation tissue is thus minimized.

METHODS.

Cortisone acetate (Merck) was administered intramuscularly to albino Wistar-strain rats weighing 120–220 g. in a daily dose of 5 mg. per rat. The appropriate operative procedure was carried out 48 hr. after the first injection. Cortisone injections were continued until the animals were sacrificed. Comparable groups of rats given no cortisone served as controls.

Operative procedure for tendon regeneration.—The right Achilles tendon of 20 cortisone-treated and 30 control rats was transected through a short skin incision, strict asepsis being observed. The severed ends were not sutured together and no attempt was made to immobilize the operated limb. Animals were killed 5, 10, 14 and 21 days after operation, 5 on each occasion. The severed tendon and intervening tissue was dissected out and fixed in 10 per cent formalin or in 80 per cent ice-cold alcohol. The blood vascular pattern was studied in some specimens by the technique of Bishton and Rogers (1950), employing a colloidal silver iodide injection. Paraffin sections were cut at 5–10μ and stained with Harris's haematoxylin and eosin, van Gieson's stain, and 0·2 per cent toluidine blue buffered to pH 5·0 (for metachromasia). The distribution of alkaline phosphatase was observed in the alcohol-fixed specimens using the method of Gomori (1939, 1941), observing the precautions which Danielli (1946) has indicated.

Operative procedure for epithelial regeneration.—Endotracheal curettage was performed on the anterior surface of the trachea in 30 cortisone-treated and 45 control rats, using a small sterile spoon curette which limited the curettage to a depth such that the elastic layer of the lamina propria was left intact. The denuded surface extended from the thoracic inlet to the larynx and measured about 2 mm. in width. Animals were killed at intervals between 4 hr. and 14 days after operation. The trachea was fixed in 10 per cent formol-saline; paraffin sections of 5–7μ thickness were cut transversely and stained with Ehrlich's acid haematoxylin and eosin, Heidenhain's iron haematoxylin and eosin, Hart's modification of Weigert's elastic stain, van Gieson's stain, Southgate's modification of Mayer's muci-carmine stain and the periodic acid-Schiff method of McManus (1946).

RESULTS.

Regenerating tendon.

Control group.—Macroscopically, the severed tendon stumps are connected by a band of tissue which in the 5-day specimens is dark and soft, apparently mainly fibrin and altered blood, but in the later stages becomes progressively paler and firmer. This connecting piece measures 5–10 mm. in length and is always considerably larger in diameter than the normal Achilles tendon. Microscopically, by the 5th day numerous spindle-shaped fibroblasts, associated with fine collagen fibres, have grown in from the surrounding connective tissue and are arranged on longitudinal strands of fibrin extending from one stump to the other. Subsequently these cells become filamentous and the collagen fibres become coarse. A few fine blood-vessels are found in the fibrin coagulum between the stumps by the 5th day, many more by the 10th and 14th days. For the most part the vessels run parallel to the long axis of the defect. Material which stains metachromatically, presumably mucopolysaccharide, is seen between the new cells and fibres, becoming progressively more intense through the 21-day period. Alkaline phosphatase is not demonstrable at 5 days, but in the 10-day specimens a faintly positive reaction is apparent in the region of the junction of

the new tissue with the old stump. In later specimens it becomes very strongly positive throughout the whole regenerating tendon.

Cortisone-treated group.—Macroscopically the intermediate piece at 5 days does not differ from that of the control group, but by the 10th day of regeneration there is a remarkable difference in its diameter. The new tendon is invariably smaller in diameter than the normal Achilles tendon. On microscopic examination no significant difference from the control group in the character or sequence of the changes is observed. Young fibroblasts and new vessels are growing into the fibrin coagulum by the 5th day (Fig. 1). Metachromatic ground substance and alkaline phosphatase are demonstrable in the 10, 14 and 21-day specimens. The production of collagenous fibres (Fig. 2) appears to be comparable to that of the controls although, since the whole tissue is thinner in the cortisone-treated animals, the absolute amount of collagen formed is less. The blood-vessels, as revealed by the injection technique, are numerous by the 10th day, and have the same general longitudinal disposition (Fig. 3) as those of the control group.

Regenerating tracheal epithelium.

Control group.—Four hours after curettage the denuded surface is covered by a fibrin clot and the underlying submucosa shows moderate inflammation. Migration of flat epithelial cells derived from the intact marginal epithelium has begun. After 8 hr. these cells have migrated for distances of about 200μ. They undermine the fibrin clot (Fig. 4), and cover the entire surface within 48 hr. Throughout the whole process the new epithelium maintains close contact with the continuous elastic layer. During the next 24 hr. this epithelium becomes stratified, 3–6 cells in depth, and the first evidence of differentiation to cuboidal epithelium appears 96 hr. after operation. Ciliated cells and goblet cells appear by the 10–14th day of regeneration. In some specimens showing pronounced submucosal inflammation epithelial differentiation is delayed.

Cortisone-treated group.—Cortisone has no influence on the rate of migration of the flat epithelial cells which spread over the elastic lamina. The time required for the complete covering of the denuded area and also for the beginning of stratification of this epithelium is the same as in the control group. However, the further differentiation of the stratified epithelial layer to cuboidal epithelium containing goblet cells and ciliated cells is somewhat in advance of that in the control specimens. By 72 hr. the new epithelium already shows evidence of differentiation into cuboidal-type cells, and within 7 days ciliated cells and goblet cells appear. By the 10th day the epithelium has a degree of maturity reached by the control group only on the 14th day. Moreover, the 14-day specimens of the cortisone-treated group display distinctly more differentiation than those of the control animals at this stage (Figs. 6 and 7). The post-operative inflammatory reaction is less severe in the cortisone-treated rats. The fibrin clot is smaller, submucosal oedema and congestion are less extensive and the inflammatory exudate contains a much smaller number of neutrophils.

DISCUSSION.

Magarey and Gough (1952) reported, and it is also our experience, that a dose of 5 mg. cortisone daily is sufficiently large to produce toxic effects in rats after several days of continuous administration. The animals lose weight and a few

of them die. In spite of this large dose, the only effects which we have observed are of a quantitative nature.

In regenerating tendon there is no effect on the time of appearance of fibroblasts, metachromatic ground substance, fibres, alkaline phosphatase or blood-vessels. The abundance of vessels is in particularly striking contrast to the reported absence of blood-vessels when the development of connective tissue in cortisone-treated animals was studied under different experimental conditions. The only positive finding is a reduction in the amount of new tissue which develops. Even so, a functionally adequate substitute tendon forms, and in this tissue all the essential characteristics of the regenerated tendon of the control group are present. The question is whether even the quantitative difference in the amount of tissue formed represents a specific effect of cortisone, for the withholding of cortisone from one group does not provide an entirely specific control. The animals of the control group do not lose weight and none dies. Thus, it could be that the diminished tendon regeneration arises from the systemic side-effects of what is a toxic dose of cortisone for the rat. To investigate this question further one might either reduce the dose of cortisone, thereby running the risk of missing a specific effect or, preferably, place the control animals on a diet which produces comparable weight loss and debility.

In epithelial regeneration cortisone does not affect the rate of epithelialisation. It lessens the inflammatory reaction (see review by Duke-Elder and Ashton, 1951), and slightly hastens the differentiation of the new epithelium. The second of these two findings may be dependent upon the first, because in control rats prominent submucosal inflammation delays epithelial differentiation.

SUMMARY.

The regeneration of Achilles tendon and tracheal epithelium has been studied in rats given 5 mg. cortisone daily and in an untreated (control) group.

The administration of cortisone results in the production of a substitute tendon histologically similar to the regenerated tendon of untreated animals but smaller in amount.

Cortisone does not alter the rate of epithelialisation of shallow tracheal wounds produced by curettage.

We are indebted to Professor G. R. Cameron, F.R.S. for suggesting this problem and for his advice and assistance in preparing this paper. This investigation was made possible by a grant to Professor Cameron from the Medical Research Council. We acknowledge generous supplies of cortisone from Messrs. Merck & Co. Inc.

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DESCRIPTION OF PLATES.

FIG. 1.—Rat tendon regeneration during cortisone administration, 5 days. New vessels and fibroblasts are growing into the fibrin coagulum. H. and E. × 200.

FIG. 2.—Rat tendon regeneration during cortisone administration, 3 weeks. H. and E. × 140.

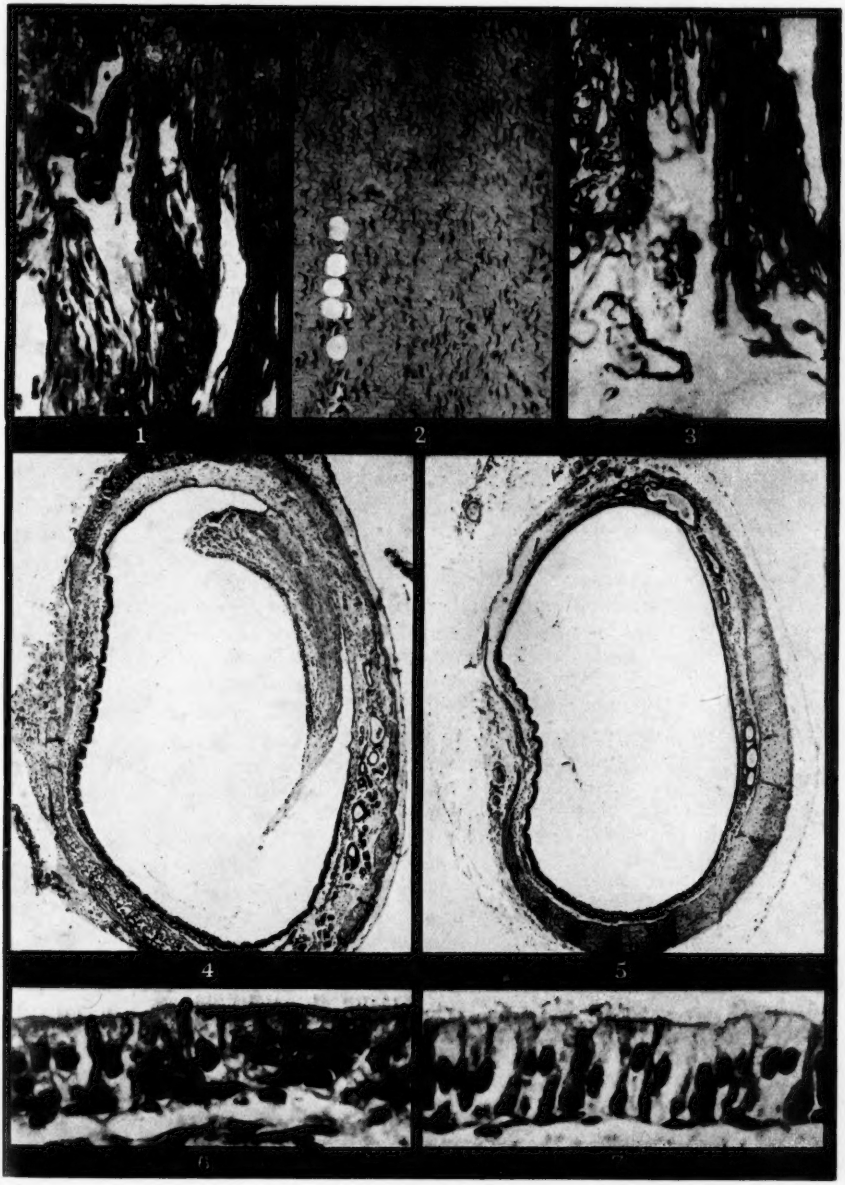
FIG. 3.—Rat tendon regeneration during cortisone administration, 10 days. Blood-vessels in the new tendon near the proximal stump (below). Colloidal silver iodide injection. × 50.

FIG. 4.—Control rat. Epithelial regeneration 24 hr. after tracheal curettage. The layer of new epithelium undermines the fibrin coagulum over the site of curettage. Inflammation is submucosal and peritracheal. H. and E. × 35.

FIG. 5.—Cortisone-treated rat. Epithelial regeneration 48 hr. after tracheal curettage. Re-epithelialisation is complete. Inflammation is slight. H. and E. × 35.

FIG. 6.—Control rat. Epithelial regeneration 14 days after tracheal curettage. The new epithelium is ciliated but not well differentiated. H. and E. × 700.

FIG. 7.—Cortisone-treated rat. Epithelial regeneration 14 days after tracheal curettage. The new epithelium is ciliated, well differentiated and approaching normal height. H. and E. × 700.



Buck and Wilhelm.

THE ENZYMIC AND LIPID HISTOCHEMISTRY OF EXPERIMENTAL TUBERCULOSIS.

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THE whole question of the different behaviour towards a tuberculous infection of animals having different degrees of sensitivity is an extremely difficult one, but the processes which follow the injection of virulent tubercle bacilli are fairly well known. It is generally agreed that the mononuclear phagocyte is the most important single cell concerned, and that in the production of the characteristic reaction certain more or less accurately identified fractions of the bacilli are essential (*e.g.*, Anderson's (1927, 1932) tuberculophosphatide, which was shown by Sabin, Doan and Forkner (1930) to produce tubercles in normal tissues). On the death of the bacilli the various fractions must be disposed of in some way, and if local breakdown is the mechanism then the enzymes concerned should be identifiable. Gerstl and Tennant (1942), investigating the hydrolysing effect on tuberculophosphatide of tissue extracts from mouse, guinea-pig and rabbit, showed that normal mouse and rabbit tissues split a far greater amount of the substrate than those of the guinea-pig. These authors suggested that two enzymes, a phosphatase and a lecithinase, take part in the breakdown of tuberculophosphatide in the body.

Rich (1951*b*) and Nungester (1951) point out that enzyme activity in mononuclear phagocytes may be of importance in host resistance, because these cells ingest and harbour the bacilli; the internal constitution and enzymic armament of these cells is thus likely to play an important part in the successful disposal of the bacilli. We have therefore studied the enzymic histochemistry of the tuberculous process in mice, rats, guinea-pigs and rabbits by modern methods. The enzymes examined were acid and alkaline phosphatase, 5-nucleotidase, phosphamidase, non-specific esterase, lipase and β-glucuronidase. At the same time the neutral fats, ester and acetal phosphatides, and fatty acids of the lesions were investigated by methods whose specificity, in some cases, overlapped.

MATERIAL AND METHODS.

A total of 12 white mice (weighing 18–22 g.), 6 rats of the Wistar strain (200–250 g.), 12 guinea-pigs (400–500 g.) and 6 rabbits (1400–1600 g.) were inoculated with the Vallée strain of bovine *Myco. tuberculosis*. The bacilli were cultivated in Dubos' liquid medium for 11 days and centrifuged in a Hopkins vaccine centrifuge at 3000 r.p.m. for 30 min. The intravenous dosage was 0·02 mg. (wet wt.)

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to mice and 1·0 mg. to rats; 0·1 mg. was injected subcutaneously in guinea-pigs and rabbits. These doses and methods of administration were chosen solely in order to obtain tuberculous lesions in every animal.

The animals were killed with coal gas in groups comprising 2 mice, 1 rat, 2 guinea-pigs and 1 rabbit, 13, 18, 31, 39, 53 and 59 days after injection. An autopsy was performed on each animal, and blocks of lungs, liver, spleen and kidneys were taken for histological and histochemical investigation. The organs of 5 mice and 3 guinea-pigs, which died spontaneously, were not used for histochemical examination as the degree of autolysis (and therefore of enzyme diffusion) could not be determined. Normal control animals of each species were killed and used for the demonstration of enzymes in normal organs.

METHODS.

Haemalum-eosin and Ziehl-Neelsen staining.

Paraffin sections 6μ thick were prepared from formalin-fixed slices of liver, spleen and kidney.

Methods for Enzymes in Frozen Sections.

It has been shown previously (Seligman, Chauncey and Nachlas, 1951; Eränkö, 1951; Burton and Pearse, 1952; Grogg and Pearse, 1952*a*, *b*) that short formalin fixation and frozen sections give excellent preservation of various enzymes in tissues. Slices of lung, liver, spleen and kidney from each animal were fixed in neutral 15 per cent formalin at 4° for 16 hr. From each block 12–15 frozen sections (15μ) were cut for the demonstration of the following enzymes:

Acid phosphatase (*pH* 5·0).—The modified azo-dye technique of Grogg and Pearse (1952*a*) was used because this method alone gave an accurate intracellular localisation of enzyme. Gomori's (1950*a*) improved lead-phosphate technique was used in all cases to confirm the topographical distribution of enzyme. With the azo-dye method incubation was carried out at room temperature for 1 hr., and with the lead-phosphate method at 37° for 1 and 4 hr.

Alkaline phosphatase (*pH* 9·2).—The coupling azo-dye technique for this enzyme first described by Menten, Junge and Green (1944) was developed further by Manheimer and Seligman (1949) and Gomori (1951). In our own attempts at improving this technique (1952*b*) it was found that the best coupling agents were the diazonium salts of 4-benzoylamino-2:5-dimethoxy-aniline, 5-chloro-*o*-anisidine and 5-chloro-*o*-toluidine. Using this technique we were able to avoid some of the diffusion artifacts produced by Gomori's (1939) calcium-phosphate method, which only gives an approximate indication of the distribution of enzyme activity. With the azo-dye method sections were incubated at room temperature for 1 hr., and with the calcium phosphate method at 37° for 1 and 4 hr.

5-*Nucleotidase* (*pH* 7·5).—The method of Pearse and Reis (1952) was used and sections were incubated at 37° for 4 hr.

Phosphamidase (*pH* 5·6).—Gomori's (1948) phosphamidase method was used, incubating sections at pH 5·6 with *p*-chloranilidophosphonic acid and lead nitrate for 4 hr. The localisation of the precipitates obtained by this method closely resembled those obtained by the lead-phosphate technique for demonstrating acid phosphatase.

Esterase (*pH* 7·5).—The histochemical demonstration of esterase was first described by Nachlas and Seligman (1949), and improved later by Gomori (1950*b*, 1952). We followed Gomori's method, using α-naphthyl acetate as substrate and diazotised *o*-dianisidine as coupling agent. The sections were incubated at room temperature for 10–15 min.

Lipase (*pH* 7·4).—Trials were carried out with Mark's (1950) modification of Gomori's (1945, 1946) method for demonstrating lipase (using Tween 60 and 80 and Product 81 as substrates). In both frozen and paraffin sections the precipitates were so patchy and coarse that further work did not seem worth while.

β-*glucuronidase* (*pH* 5·2).—The 8-hydroxyquinoline glucuronide method of Friedenwald and Becker (1948) modified by Burton and Pearse (1952) was used, with incubation at 37° for 4 hr.

Controls for enzyme methods.

For each experiment control sections were incubated in the absence of substrate. In some cases an additional control containing substrate plus the specific inhibitor was employed.

Demonstration of Enzymes in Paraffin Sections.

Although we regard cold formalin fixation and frozen sections as essential for the best results with all the above enzyme methods, a comparison with paraffin sections was also made. Slices of tissue 2–3 mm. thick were fixed in two or three changes of cold absolute acetone at 4° for 24 hr. They were then transferred consecutively to absolute alcohol, absolute alcohol-ether with one or two changes, and thence to 1 per cent celloidin. After draining off any excess of the latter the blocks were hardened in chloroform, cleared in benzene and embedded in paraffin wax. Sections were cut at 5μ and mounted on albuminised slides. Heating on the hot stage was avoided by drying the slides for 30 min. in a 37° incubator. Slides and blocks were then stored in the cold until required for incubation. This process was carried out after removing the wax from the slides by immersion in light petroleum, followed by absolute acetone and rinsing in running water. The incubation times were usually 2–3 times greater than those used for the frozen sections.

Method for Lipids.

McManus' (1946) *method.*—Slices of tissue were fixed in McManus' formalin-calcium-cobalt mixture for 1 week. The material was dehydrated in alcohol, cleared in benzene, embedded in paraffin, cut in 6μ sections and stained with Sudan black B. By this method formol-fixed lipid, such as phosphatide or cerebroside, can be demonstrated. Only positive results were considered to be of any value.

Baker's (1946) *method.*—Material fixed in formalin-calcium-cobalt mixture for 1 week was used for Baker's acid haematein and pyridine extraction methods.

Fischler's (1904) *method.*—Tissue was fixed in 15 per cent neutral formalin for 48 hr. and cut in frozen sections of 15μ. Fischler's method was used for demonstrating fatty acids, because it is the only method available for this purpose. Lison (1936) regarded the technique as non-specific, and negative results are certainly not significant.

Feyrter's (1936) *method.*—The " enclosure staining " method (tartaric acid-thionin) was applied to cold formalin-fixed frozen sections (48 hr. and 14 days). This reaction demonstrates lipids of the phosphatide class.

Plasmal reaction (modified).—Slices of tissue were fixed in 15 per cent neutral formalin for 16 hr. Frozen sections were cut and treated with 20 per cent hydroxylamine hydrochloride in saturated aqueous sodium acetate for 20 min., in order to block existing aldehydes before oxidation with $HgCl_2$. Further steps were carried out as described by Hayes (1949).

Reactions for neutral fat.—With formalin fixed (48 hr.) frozen sections several techniques for neutral fat were used : Sudan III/IV in 70 per cent alcohol, Oil Red 4B or Oil Red 0 in *iso*propanol solution (Lillie, 1948), Fettrot (Ciba) and Sudan black B in 70 per cent alcohol, and Fettrot (Ciba) and Sudan black B as saturated solutions in propylene glycol (Chiffelle and Putt, 1951).

RESULTS.

Our most striking results concern the distribution of acid and alkaline phosphatase and esterase in the lesions, and those in each species will be described separately. At the same time the morphological features of the lesions, which form the essential background to the histochemistry, are necessarily described. The results of the studies in lipid histochemistry will be reported as a whole, in a separate section. It must be clearly stated at this point that we regard the mononuclear phagocytes of the tissues, the monocytes of the blood-stream and the epithelioid cells of the tuberculous lesions as variations of a single cell type, in agreement with the views of Lewis (1925–1926) and Lewis and Lewis (1926). We would also emphasize the point that the histochemical results for acid and alkaline phosphatase and esterase, recorded below, are entirely based on coupling

azo-dye techniques applied to cold formalin-fixed frozen sections. Gomori's (1950*a*) lead-phosphate technique (acid phosphatase) and his 1939 calcium-phosphate technique (alkaline phosphatase) were only used for comparison and control.

Mouse.

Morphology.—In all mice we found miliary tuberculosis with large tubercles in the lungs. The livers and spleens were always enlarged. Microscopy revealed no great differences between one animal and another in spite of the varying length of survival after infection. The tubercles in the lungs showed a variable amount of coagulative necrosis surrounded by a dense infiltration of mononuclear phagocytes (foamy cells), a few lymphocytes and an occasional polymorph. Some aggregations of mononuclear phagocytes were scattered throughout the liver and spleen. There was an area of coagulative necrosis in the kidney of one mouse. Acid-fast bacilli were present, singly or in clumps, in most of the lesions.

Our findings corresponded to those reported by Youmans and McCarter (1945) for their control mice in streptomycin experiments.

Acid and alkaline phosphatase and esterase.—A strong acid phosphatase reaction was observed in the cytoplasm of the mononuclear phagocytes forming the tuberculous nodules in the liver and spleen as well as in the mononuclear phagocytes and large foamy cells in the lung lesions (Fig. 1). In the liver and the spleen the mononuclear phagocytes in the tuberculous lesions contained a far stronger acid phosphatase than did the adjoining normal tissue cells. Alkaline phosphatase was not demonstrated in the lesions. An esterase was demonstrated in some of the mononuclear phagocytes in the tubercles in the lungs but, on the whole, esterase activity in the lesions was less than in normal lung, in which the septal cells contained a considerable amount of esterase.

EXPLANATION OF PLATES.

FIG. 1.—Frozen section, mouse lung. Coupling azo dye technique. Shows mononuclear phagocytes in and around a tuberculous focus staining deep red (acid phosphatase). Mayer's haemalum, × 145.

FIG. 2.—Frozen section, rat lung. Coupling azo dye technique. Shows mononuclear phagocytes as in the mouse. Mayer's haemalum, × 145.

FIG. 3.—Frozen section, guinea-pig lung. Coupling azo dye technique. The necrotic centre of a tuberculous lesion is shown, filled with red-staining acid phosphatase. Mayer's haemalum, × 40.

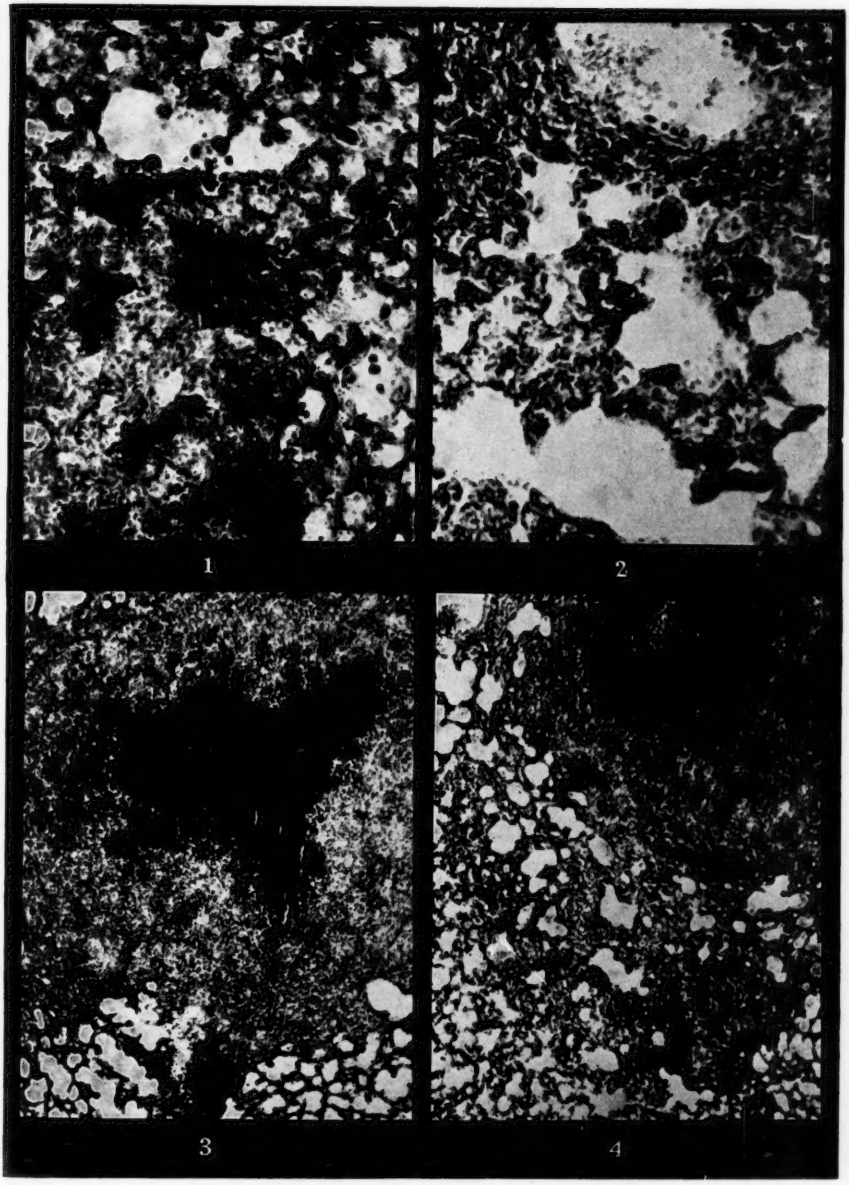
FIG. 4.—Frozen section, guinea-pig lung. Coupling azo dye technique. Shows alkaline phosphatase, free and within the cytoplasm of polymorphs, in the necrotic centre of the lesion and around it. Mayer's haemalum, × 40.

FIG. 5.—Frozen section, rabbit lung. Coupling azo dye technique. Shows acid phosphatase-laden mononuclear phagocytes congregating around a small lymphoid follicle in the wall of an artery. Mayer's haemalum, × 145.

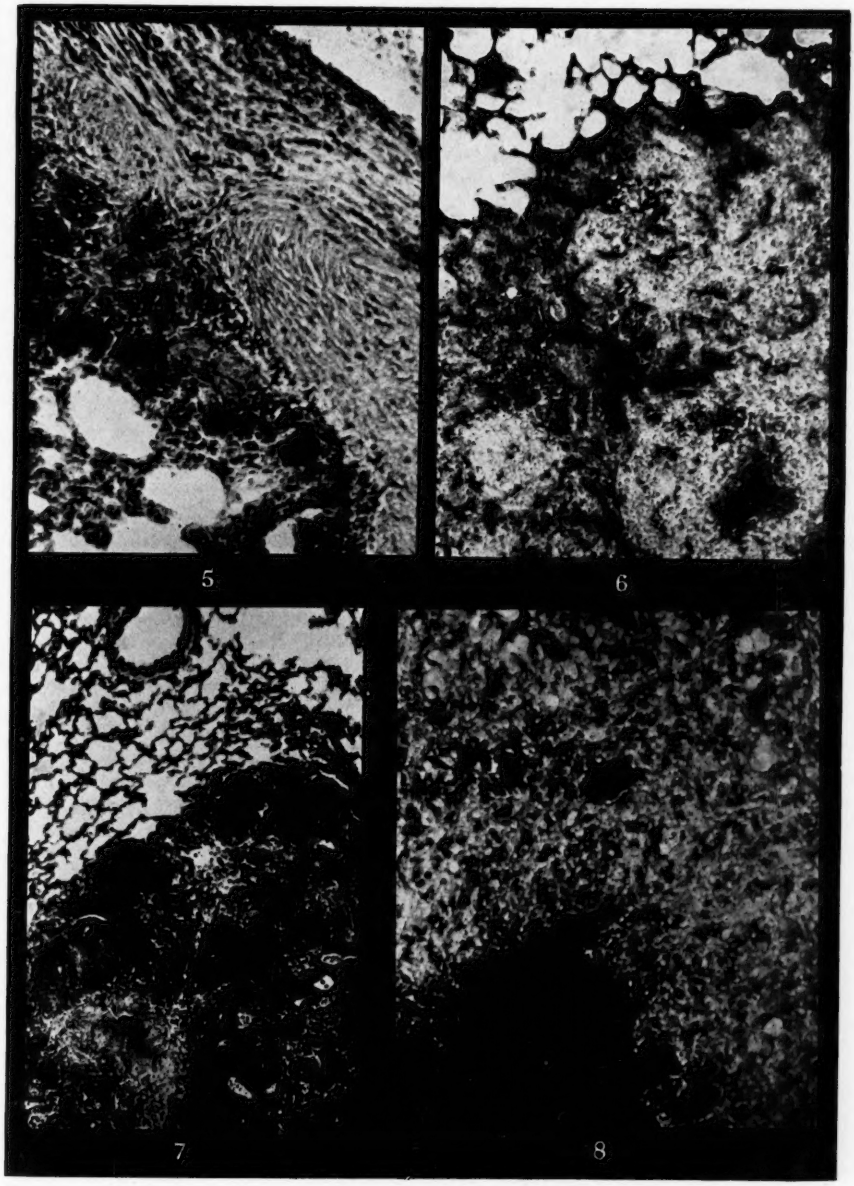
FIG. 6.—Frozen section, rabbit lung. Coupling azo dye technique. Shows alkaline phosphatase present in tuberculous lesions only in the polymorphs and in the walls of small blood vessels. Mayer's haemalum, × 53.

FIG. 7.—Frozen section, rabbit lung. Coupling azo dye technique. Shows esterase-laden mononuclear phagocytes within the substance of a tuberculous focus. The pale staining necrotic centre contains some free esterase. Mayer's haemalum, × 40.

FIG. 8.—Frozen section, guinea-pig spleen. Coupling azo dye technique. Large amounts of intra- and extra-cellular acid phosphatase present in the necrotic zone, and a smaller amount in the giant cell. Nearly all the remaining tissue is composed of phosphatase-negative epithelioid cells. Mayer's haemalum, × 145.



Grogg and Pearse.



Grogg and Pearse.

We found that the monocytes of the blood stream in tuberculous and control animals, gave a positive reaction for acid phosphatase and occasionally for esterase; the polymorphs were negative for acid and alkaline phosphatase but occasionally contained a weak esterase.

Rat.

Morphology.—No gross tubercles were found, but the spleen and liver were enlarged and the lungs consolidated. Histologically, miliary nodules formed only by mononuclear phagocytes were constantly found in the lung, liver and spleen; a few lymphocytes and single polymorphs were occasionally seen in some fresh lesions. In the lung nodules some of the mononuclear phagocytes were vacuolated or foamy. Generally speaking, the tuberculosis was purely proliferative and without necrosis. Acid-fast bacilli were present in all lesions. The histological features, as well as the numbers of bacilli, were similar to those observed by Wessel (1941) and Grogg (1952).

Acid and alkaline phosphatase and esterase.—The mononuclear phagocytes in the lesions contained a large amount of acid phosphatase, a fact already reported by Grogg (1952) using the standard Gomori technique on paraffin sections. Fig. 2 illustrates the presence of the enzyme in mononuclear cells in the neighbourhood of a lesion in the lung. Neither alkaline phosphatase nor esterase was demonstrated in cells in the lesions.

Among the circulating leucocytes of tuberculous and control animals many polymorphs contained alkaline phosphatase, and some contained a weak acid phosphatase. The monocytes showed a strong positive reaction for acid phosphatase in their cytoplasm and some contained a fairly strong esterase. Only a very weak reaction for esterase was given by the polymorphs.

Guinea-pig.

Morphology.—In all animals autopsy revealed progressive tuberculosis in lungs, liver, spleen and lymph-nodes. The organs were greatly enlarged and stuffed with tubercles. Yellow infarcts were seen in some spleens. Microscopy revealed an exudative tuberculosis in all animals. Fresh lesions appeared small and consisted mainly of polymorphs; other tubercles had a necrotic centre which was surrounded by polymorphs, epithelioid cells, giant cells and lymphocytes, a picture similar to that seen in human tuberculosis. Acid-fast bacilli were found in the lesions.

Acid and alkaline phosphatase and esterase.—Some acid phosphatase was observed in the polymorphs, but none in the epithelioid cells of the lesions. The necrotic centres of the lesions, however, containing débris of polymorphs and amorphous material, gave the strongest positive reaction found in any animal in this series (Fig. 3), and in no other was an extracellular acid phosphatase detected. Although acid phosphatase was not demonstrated in the epithelioid cells, the cytoplasm of the giant cells stained positively (Fig. 8). Ingestion of polymorphs by giant cells was seen in some of these cells, and it is suggested therefore that the acid phosphatase reaction of the giant cells is partly or entirely due to ingested polymorphs, although it is not possible to exclude the presence of an intrinsic acid phosphatase. A positive reaction for alkaline phosphatase was observed in the cytoplasm of the polymorphs, and its distribution inside the earliest lesions

corresponded with the distribution of these cells. In the older lesions free (extracellular) enzyme was present in small quantities (Fig. 4), but never in large amounts, as in the case of acid phosphatase. Esterase was shown in some of the giant cells but, on the whole, it could not be demonstrated intracellularly in the tuberculous lesions. The necrotic centres of the lesions contained a weak extracellular esterase.

All the polymorphs within the blood-vessels contained acid and alkaline phosphatase. No strongly positive reaction for esterase was observed in the blood cells, but some of the polymorphs were weakly positive.

Rabbit.

Morphology.—Lung tubercles of variable size were present in all the animals. On histological examination these lesions were seen to consist either of necrotic centres surrounded by polymorphs, with infiltrations of mononuclear phagocytes and a few lymphocytes, or of mere accumulations of mononuclear phagocytes with foamy appearance together with a few lymphocytes.

Acid and alkaline phosphatase and esterase.—The mononuclear phagocytes contained a moderately strong acid phosphatase (less strong than in rat or mouse). The monocytic infiltrations were thus strongly stained, whereas the necrotic centres of the lesions remained colourless (Fig. 5). Alkaline phosphatase usually occurred only in the cytoplasm of polymorphs and in the vascular endothelia within the lesions, but free alkaline phosphatase was present in the necrotic areas (Fig. 6). Single groups of mononuclear phagocytes within the lesions gave a strong positive reaction for esterase in their cytoplasm. These black-stained cells are shown in Fig. 7.

Most but not all of the polymorphs in the vessels contained a strong alkaline phosphatase. A weaker acid phosphatase was present in some of the polymorphs and a strong acid phosphatase in the majority of the monocytes. The blood monocytes gave a strong esterase reaction.

Observations on 5-nucleotidase, phosphamidase and β-glucuronidase.

5-Nucleotidase.—The results observed in tuberculous lesions stained by the method for 5-nucleotidase were very similar to those produced by the alkaline phosphatase (calcium-phosphate) technique; *i.e.*, accumulations of polymorphs were stained in the lungs and spleens of guinea-pigs and rabbits. There was no striking difference within the lesions themselves that could be attributed to the activity of 5-nucleotidase alone.

Phosphamidase.—Using Gomori's (1948) technique for phosphamidase we observed precipitates in polymorphs and caseous material inside tuberculous lesions in the guinea-pig. The distribution was similar to that of acid phosphatase (lead-phosphate technique).

β-glucuronidase.—A strongly positive reaction was seen in the foamy cells (mononuclear phagocytes) of lung lesions in the rat.

Results of staining methods for lipids.

Using Sudan black B, Fettrot (Ciba), Sudan III/IV and Oil Red, deep staining of the foamy cytoplasm of mononuclear phagocytes in the lung lesions of rats and mice was obtained. This reaction was not present in the large mononuclear phagocytes of lung lesions in rabbits. A faint staining of fatty droplets

was seen in polymorphs and in the epithelioid cells of caseous lesions in guinea-pigs.

We observed some staining of the foamy cells in tuberculous lesions in rats, using Baker's (1946) acid haematein method, negative controls being obtained with pyridine extraction. With the modified plasmal reaction a weak positive result was obtained in the foamy cells and strongly positive inclusions were seen in small groups of monocytes outside the lesions. Feyrter's (1936) " Enclosure staining " method gave a positive result in the foamy cells after 48 hr. fixation in formalin but a negative one after 14 days' fixation. This suggests that the phosphatide demonstrated in the first instance was of low molecular weight and extractable from the tissues by comparatively short sojourn in formalin. Fatty acids could not be demonstrated in any of the tuberculous lesions.

In Table I the presence of enzymes in the lesions, and their situations therein, are summarised. Table II records the presence of acid phosphatase, alkaline phosphatase and esterase in the polymorphs and monocytes of the blood stream (tuberculous and normal control animals).

TABLE I.—*Enzymes in Tuberculous Lesions.*

Animal species.	Site.	Acid phosphatase.	Alkaline phosphatase.	Esterase.
Mouse	Mononuclear phagocytes	+	0	0
Rat	Polymorphs	0	+	0
	Mononuclear phagocytes	+	0	0
Guinea-pig	Necrotic material	+	+	0
	Polymorphs	+	+	0
	Mononuclear phagocytes	0	0	0
	Giant cells	+	0	+0
Rabbit	Necrotic material	+0	+	0
	Polymorphs	+0	+	0
	Mononuclear phagocytes	+	0	+

+, enzyme demonstrated by the histochemical methods used.
0, enzyme not demonstrated by the histochemical methods used.
+0, enzyme present in smaller amounts or in a proportion of cells only.

The Effects of Streptomycin and Isonicotinic Acid Hydrazide on the Enzymes in Tuberculous Lesions in Guinea-pigs.

In order to ascertain the effects of anti-tuberculous drugs on the enzymes present in tuberculous lesions, two infected animals were treated with streptomycin, two with isonicotinic acid hydrazide and two served as controls. Both drugs were given in daily doses of 10 mg. for 6 days a week over a period of 3 weeks, commencing on the 21st day after infection.

At autopsy all animals showed miliary tuberculosis, but histological examina-

tion revealed no significant morphological differences in treated and untreated animals. The treated animals showed fewer polymorphonuclear leucocytes, but there was no apparent difference in the degree of fibrosis. The distribution of acid and alkaline phosphatase and esterase in the lesions was similar in treated and untreated animals.

The results of this experiment support our hypothesis as to the rôle played by the enzymes in the mechanism of natural resistance. If anti-tuberculous drugs act on tubercle bacilli in the manner attributed to them, they cannot cause any alteration in the enzyme activity when this is related to the natural resistance of the animal.

TABLE II.—*Enzymes in Blood Cells.*

Animal species.	Site.	Acid phosphatase.	Alkaline phosphatase.	Esterase.
Mouse	Polymorphs	0	0	+0
	Monocytes	+	0	+
Rat	Polymorphs	+0	+	+0
	Monocytes	+	0	+0
Guinea-pig	Polymorphs	+	+	+0
	Monocytes	0	0	0
Rabbit	Polymorphs	+0	+	0
	Monocytes	+	0	+

+, enzyme demonstrated by the histochemical methods used.
0, enzyme not demonstrated by the histochemical methods used, but the possibility of its presence in similar cells under different conditions is not excluded.
+0, slight staining of cells occasionally present.

DISCUSSION.

In comparing the histochemical findings in the mononuclear phagocytes of different animals with their varying degrees of resistance to infection with *Myco. tuberculosis* it is tempting to see possible correlations. The mouse and rat have greater natural resistance to tuberculosis than the guinea-pig and rabbit (Rich, 1951*a*), and our observations show that the mononuclear phagocytes of the more resistant animals contain a strong acid phosphatase, whereas in the susceptible guinea-pig they do not. The results obtained in the rabbit unfortunately prevent complete correlation of mononuclear acid phosphatase with resistance to tuberculosis. However, the strong esterase present in rabbit mononuclear phagocytes, and absent from those of the mouse and rat, may provide a clue to further differences between the rabbit and the more resistant species.

It is known that tuberculophosphatide induces tuberculoid reactions in animals (Sabin *et al.*, 1930), but the amount of phosphatide necessary to produce epithelioid cells and giant cells is much larger than that present in natural infection (Rich, 1951*c*). (Moreover, Ungar, Coulthard and Dickinson (1948) have shown that synthetic fatty acids elicit tubercle-like reactions). In spite of this objection we suggest that tuberculophosphatide is a substrate for the acid phosphatase of the mononuclear phagocytes since Rous (1925), using vital dyes, showed that the reaction of these cells in the mouse and rat is acid. According to our results, therefore, the mononuclear phagocytes of susceptible species like the guinea-pig would be unable to metabolise tuberculophosphatide. This reasoning agrees with the results of Gerstl and Tennant, described in the introduction. It is obvious,

however, that acid phosphatase could play its part in the intracellular metabolism of carbohydrates and lipids without acting on tuberculophosphatide and without having significant effect on the mechanism of natural resistance.

It has long been known that the initial reaction to tubercle bacilli introduced into the tissues of resistant or susceptible animals is a polymorphonuclear one (Borrel, 1893). A further mechanism, which is observed to operate in susceptible animals, is the breakdown of polymorphs to form necrotic centres to the lesions. In the guinea-pig large amounts of acid and alkaline phosphatase are released into the caseous material by this process (Fig. 3 and 4), and it is probable that they there play a part in calcification of the lesions. It is probable that other enzymes, not at present histochemically demonstrable, escape from the dying polymorphs at the same time. Among these the various proteases, known to be present in leucocytes, might be responsible for the outward spread of the lesions in susceptible animals.

The studies in lipid histochemistry which we made have contributed little to our understanding of the tuberculous process except in suggesting the presence of a diffusible phosphatide, as well as the large amount of ordinary phosphatide, cholesterol and neutral fat known to be present. Levaditi and Vaisman (1949), using Sudan III and Sudan IV, stained fatty granules in tuberculous lesions in the lungs of experimental animals, and ascribed to the foamy phagocytes a special rôle in the defence against tubercle bacilli. Grundland, Bulliard and Maillet (1951) demonstrated phosphatide in tuberculous giant cells by an acetone-cadmium method, and cholesterol with a modified digitonin method. No conclusions as to the significance of either were published. Using chemical methods Cmelik (1952) found a considerable amount of lipid in tuberculous pus. Of the total, 25 per cent was identified as phospholipid and 30 per cent as cholesterol. The extracted lipids gave a positive plasmal reaction, corresponding to our histochemical finding in tuberculous rat lungs.

If our hypothesis is correct, effective chemotherapy should not cause any change in enzyme activity, and the small scale therapeutic experiment described did not reveal any such change.

SUMMARY.

Mice, rats, guinea-pigs and rabbits were inoculated with bovine *Myco. tuberculosis*, and killed at intervals, the lesions in lung, liver and spleen being studied by histochemical methods for enzymes and lipids.

Acid phosphatase was shown to be the most important single enzyme (of those demonstrated), being present in large amounts in the mononuclear phagocytes of resistant animals.

In the guinea-pig the mononuclears contained no acid phosphatase, but large amounts of this enzyme were found in the necrotic lesions.

Since extracellular enzymes were never found in the lesions of resistant animals, the spread of tuberculosis in susceptible animals may be due to proteolytic or other enzymes derived from dead polymorphs.

The distribution and activity of acid and alkaline phosphatase and esterase in tuberculous lesions in the guinea-pig were unaltered by treatment with streptomycin or isonicotinic acid hydrazide.

The phosphatides of tubercle bacilli are suggested as one substrate for the acid phosphatase of the mononuclear phagocytes, and it is supposed that the

enzymic armament of these cells is a contributory factor in the mechanism of natural resistance to tuberculosis.

We should like to thank Professor J. H. Dible for his interest and encouragement, and Dr. D. A. Mitchison for his assistance in the preparation of the cultures and in inoculating the animals. We are grateful to Mr. E. V. Willmott for taking the photomicrographs and to Mrs. P. Carter for technical assistance. For a supply of isonicotinic acid hydrazide we are indebted to Organon Laboratories Ltd.

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HISTAMINE RELEASE FROM THE PERFUSED LUNG OF THE GUINEA PIG BY SEROTOXIN (ANAPHYLATOXIN).*

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THERE are two theories of the mechanism of anaphylaxis, one cellular and the other humoral. The first was formulated by Dale (1913, 1920, 1929), and held that the cells were the site of liberation of the anaphylactic poison (histamine). The humoral theory, developed by Friedberger (1909, 1911), Bordet (1913), Keysser and Wassermann (1911) and Nathan (1913) pointed to the blood as the source of a toxic principle, the anaphylatoxin, which was generated by contact with a specific precipitate or substances such as kaolin, barium sulphate, agar, starch, or inulin. Since anaphylatoxin prepared by treating serum with agar did not contain enough histamine to kill a guinea-pig, and did not regularly produce a stimulating effect on the isolated uterus, Dale and Kellaway (1922) decided that it could not be a true anaphylactic poison.

Recently the histamine theory has been preferred to the anaphylatoxin theory because of the experiments of Bartosch, Feldberg and Nagel (1932), Daly, Peat and Schild (1935), Ungar and Parrot (1936) and Schild (1939) in which it was conclusively shown that direct contact of the antigen with washed guinea-pig lungs and several other tissues was enough to result in the release of histamine. However, the amounts of histamine that can be liberated by perfusion of the guinea-pig lung with Tyrode solution containing antigen have always been very small, 0·4–4·0 μg. in the experiments of Bartosch *et al.* (1932) and 0·5–12·0 μg. in those of Daly *et al.* (1935).

Recently Hahn and Oberdorf (1950) showed that antihistaminics prevented the shock produced in the guinea-pig by anaphylatoxin which had been prepared by incubation of normal guinea-pig serum with inulin. This led us to study the mechanism by which anaphylatoxin produces its effects. We want to suggest that it is a powerful histamine-releasing agent and hope thus to combine the cellular and the humoral theories of anaphylaxis. Since the final mediator of the shock (histamine) comes from the tissues and the intermediate releasing agent (anaphylatoxin) is a blood-borne principle, a general theory of anaphylaxis might embody both views as parts of the same phenomenon (Rocha e Silva, Aronson and Bier, 1951 ; Rocha e Silva, 1952).

MATERIALS AND METHODS.

Serotoxin or "activated serum" was prepared according to Bordet (1913) by incubating guinea-pig serum with 0·2 ml. of a 0·5 per cent suspension of agar

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per ml. serum. After 1 hr. at 38° the material was centrifuged for 15 min. to remove most of the agar in suspension. That part of the agar which remained in the centrifugate precipitated when left in the ice box for a day. No significance can be attached to these traces of agar in the "activated serum"; even if a few ml. of the original agar suspension were injected into the guinea-pig lung no pharmacological effect could be observed. The agar used in these experiments was a fairly pure specimen, containing only 0·2 mg. N per 100 g. This is the N content given by Bordet and Zunz (1915) for purified agar. The method of

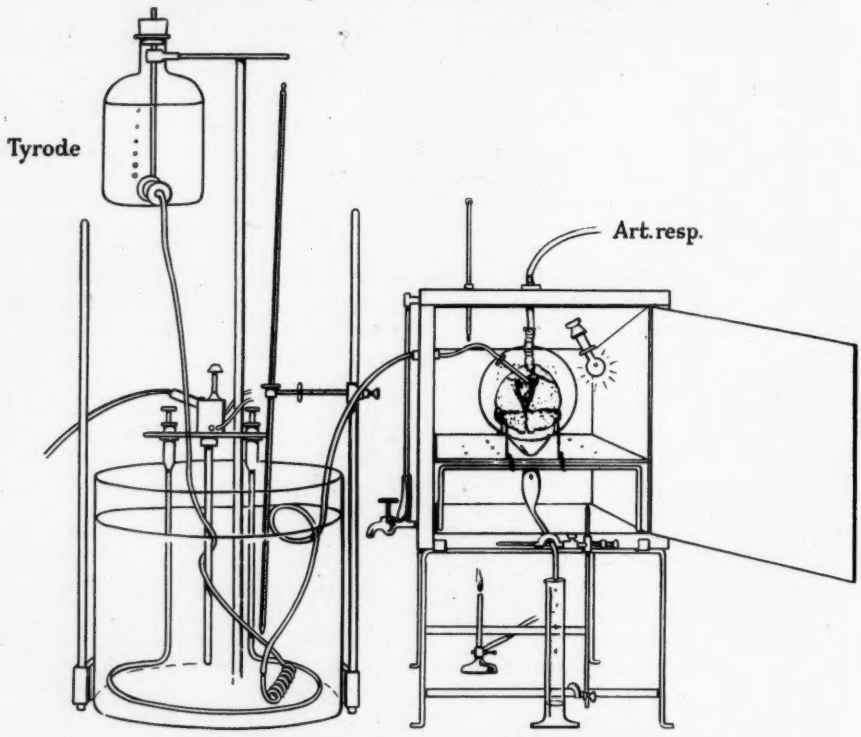

FIG. 1.—Apparatus used for perfusion of the guinea-pig's lung.

Bordet and Zunz to reduce N in agar was applied but failed to lower the N content any further.

Perfusion of the lungs was performed as follows: Guinea-pigs weighing 450–600 g. were anaesthetised with ether. The trachea was cannulated and connected with an artificial respiration pump (10 to 12 strokes per minute) and the thorax was opened. Just before inserting the perfusing cannula into the pulmonary artery the animal was bled by cutting through the abdominal vessels. After tying the cannula and washing the lung free of blood as completely as possible, both lungs were transferred to a watch-glass (12 cm. diam.), which was maintained over a funnel inside a cabinet at 37°–38°, as indicated in Fig. 1. The Tyrode

solution passed through a coil in a waterbath at 38–40° from a Mariotte's flask kept at a height of 80 cm. which secured a perfusion rate of 15 to 25 ml./min. With the fast rate of perfusion the lung was washed out rapidly and different injections could be made at a few minutes' interval. The perfusates were tested until no traces of histamine could be detected. They were collected every minute and the rate of perfusion checked all the time. Rhythmic inflations of the lungs with air at the rate of 4 to 6 per min. helped to maintain the perfusion flow. Four ml. of the activated serum were then injected and the histamine content

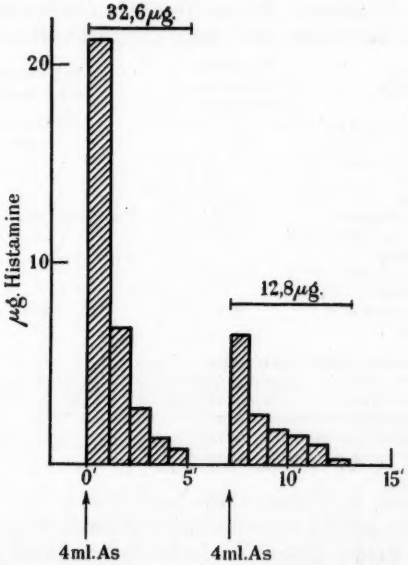

FIG. 2.—Histamine release from a guinea-pig lung by two injections into the cannula of the same amount of normal guinea-pig serum incubated with agar for 30 min. at 38°. Abscissae: time in minutes.

measured until only traces of histamine could be detected in the perfusates. The injection of 4 ml. of activated serum was then repeated and the release of histamine followed up until no more histamine could be found in the perfusates. Usually 4–6 min. was the interval necessary to collect the total histamine released after each application of activated serum.

The estimation of histamine in the perfusates was done on the guinea-pig ileum suspended in a 10 ml. chamber in a Dale's apparatus, using as a standard a histamine dihydrochloride dilution of $0{\cdot}5 \times 10^{-6}$ of the base. All histamine estimations were calculated as the base. The perfusates were assayed directly and the histamine was not specially extracted. The histamine in the activated serum was estimated separately and never attained levels sufficient to change the significance of the results. Thus in 4 ml. of guinea-pig serum no more than 0·2 to 0·5 μg. were usually found. In ox blood only negligible amounts of histamine were present.

RESULTS.

Release of histamine by activated guinea-pig serum.

As shown in Table I, the normal serum of the guinea-pig activated by incubation with agar for 1 hr. releases considerable quantities of histamine when perfused through the isolated lung of the guinea-pig. Fig. 2 shows the output of histamine following two injections of 4 ml. activated serum (Exp. 2). In the first minute of perfusion 21·3 μg. of histamine could be found in the collected

TABLE I.—*Histamine Release from the Guinea-pig Lung by Perfusion with Agar-Anaphylotoxin.*

	1st injection (4 ml. serum).	Histamine released (μg.).	2nd injection (4 ml. serum).	Histamine released (μg.).
1.	Normal (non-activated)	0	Activated	21·4+
2.	Activated	32·6	Same	12·8
3.	Dialysed (24 hr.), then activated	15·1	„	8·9
4.	Activated	25·6	—	—
5.	Activated, then dialysed	10·4	Dialysed, then activated	23·9
6.	Heated (30 min. at 56°), then activated	22·8*	„ „ „	103·3+
7.	Heated (60 min. at 60°), then activated	Traces	—	—
8.	Same serum as 7, activated (same lung as 7)	14·3	Same	9·7

" Activated " = serum treated with agar.
" Dialysed, then activated " = serum submitted to dialysis before treatment with agar.
" Activated, then dialysed " = incubated with agar for 24 hr. before dialysis.
" Normal " = untreated guinea-pig serum.
" + " = perfusion slow (about 5 ml./min.).
" * " = „ very slow (about 2–3 ml./min.).

perfusate. In 2–6 min. of perfusion the histamine in the perfusates diminished sharply, and before the second injection the perfusates were practically histamine-free. As a rule the second injection of exactly the same amount yielded about one-half of the total histamine released on the first occasion. Table II shows a comparison of the means of total histamine released in three experiments in which the same material was injected twice into the lung.

TABLE II.—*Two Successive Injections of Identical Materials in 3 Different Guinea-pig Lungs.*

Guinea-pig.	Material injected (4 ml.).	1st injection (μg. histamine).	2nd injection (μg. histamine).	Differences (μg. histamine).
1.	Activated serum	32·6	12·8	19·8
2.	Dialysed activated serum	15·1	8·9	6·2
3.	Activated serum	14·3	9·7	4·6
Averages		20·7	10·4	10·2

Dialysis of the serum performed before activation did not alter the result. In one experiment (Table I, Exp. 6) dialysis for 24 hr. before activation appeared to increase the resulting output; not less than 103·3 μg. histamine were released in the course of 10 min.; however, in other experiments (Table I, Exp. 3 and 5) previous dialysis did not produce any appreciable effect on the course of activation. Dialysis *after* activation, as performed in Table I, Exp. 5 (second injection),

appeared to reduce the activity, but this activated serum was left for 24 hr. at 5° in the cold room and spontaneous inactivation might have occurred.

Effect of temperature on the activation of serum by agar.—As shown in Table I, heating the serum for 60 min. at 60° *before* the activation with agar completely destroyed its capacity to be activated; however this was not so after heating at 56° for 30 min. When heated *after* activation the activated principle was somewhat more resistant. A temperature of 60°–69° for 90–120 min. reduced to less than one-half the releasing activity but did not destroy it completely. Table III shows 5 pairs of experiments in 10 different guinea-pigs in which the

TABLE III.—*Effect of Heating Activated Serum on Histamine Release.*

Guinea-pig.	Unheated activated serum (μg. histamine).	Guinea-pig.	Serum activated then heated (μg. histamine).	Guinea-pig.	2nd injection: activated unheated serum (μg. histamine).
5.	21·4	12	9·0 (1 hr. at 60°)	12	13·0
8.	32·6	13	14·1 (2 „ „)	13	19·1
2.	25·6	14	3·7 (1½ „ 65°)	14	8·0
11.	14·3	15	11·6 (1½ „ „)	15	9·4
16.	26·6	17	11·8 (1½ „ 69°)	17	15·6
Averages	$\bar{x}_1 = 24·1$	$\bar{x}_2 = 10·04$			13·0
Dif.	$\bar{x}_1 - \bar{x}_2 = 14·06$	$sd = \sqrt{11·37} = 3·37$; $t = 14·06/3·37 = 4·17$; $d.f. = 8$; $P < 0·01$ HS.			

release of histamine by activated serum is compared with serum that was activated and heated. As shown in the Table, the average for the first injections (total output) dropped from 24·1 μg. to 10·04 μg. histamine. The difference was found to be highly significant ($P < 0·01$).

In the experiment presented in Table III we did not go beyond 69°, to avoid coagulation of the serum proteins. In two separate experiments, however, after activation the serum was treated with 2 vol. ethyl alcohol heated at 65° for 15 min., and the filtrate was dried *in vacuo* and extracted with water. On passage through the lungs this material was completely inactive, showing that the treatment either destroyed the releasing agent or that this was not soluble in hot ethyl alcohol.

Effect of citrate on activation of serum by agar.

If instead of serum citrated plasma was used, no activation could be observed after incubation with agar for 1 hr. In the experiments shown in Fig. 3, 4, 5 and 6 ox instead of guinea-pig blood was used; ox blood yields a less active material, but clear-cut results could be obtained by comparing blood samples collected with 20 per cent of a 3·8 per cent solution of trisodium citrate with samples that were left to clot spontaneously. Both citrated plasma and serum were incubated for exactly the same time and under identical conditions. In some experiments citrate was added to the serum before addition of agar and also after activation and removal of agar. As shown in Fig. 3, 4 and 5, citrate hindered activation when added to serum before its activation by agar; added afterwards citrate did not prevent the release of histamine from guinea-pig lungs.

In order to check whether the effect of citrate could be due to removal of

Ca^{++}, the citrated blood or plasma or serum were recalcified after 12 to 15 min. contact with the citrate. The concentration of citrate used was about 0·025 M and a slight excess of $CaCl_2$ (0·04 M) was added. After coagulation of the plasma

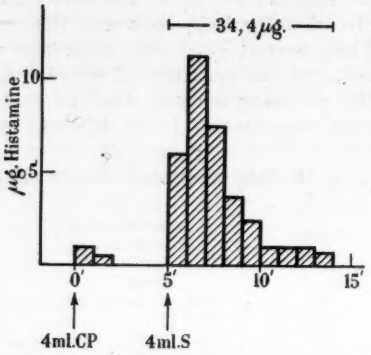

FIG. 3.—Histamine released from a guinea-pig lung by perfusion with citrated plasma (CP) and serum (S) both incubated with agar for 30 min. at 38°.

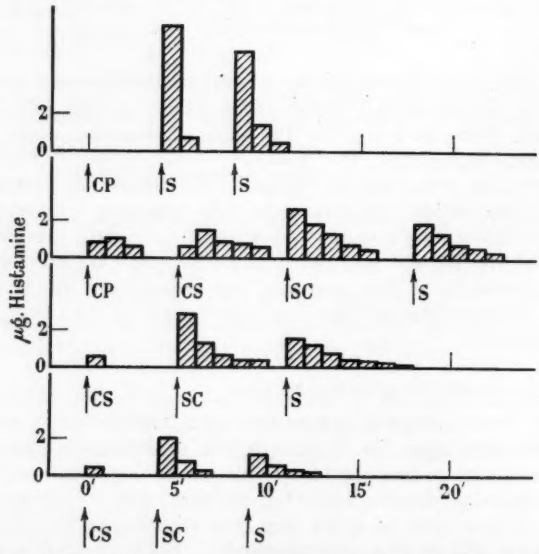

FIG. 4.—Histamine released from the lungs of 4 guinea-pigs by perfusion with citrated plasma (CP), citrated serum (CS), serum citrated after incubation (SC) and normal serum (S): all incubated with agar for 1 hr. at 38°. Abscissae: time in minutes.

the clot was removed by centrifugation and the recalcified material incubated with agar in the usual way. The results are shown in Fig. 6. If citrate is added to blood and the plasma, obtained by centrifugation, is submitted to the action of agar, it will not show any capacity of releasing histamine from the perfused

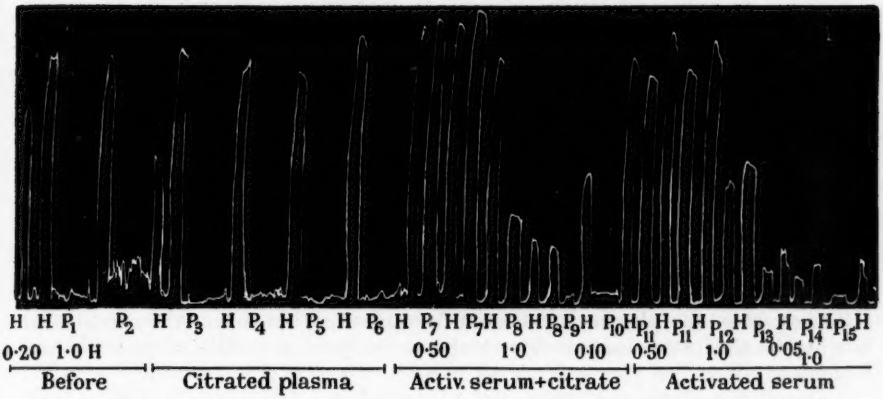
H H P1 P2 H P3 H P4 H P5 H P6 H P7 H P7H P8 H P8P9H P10HP11 H P11 H P12 H P13 H P14HP15H
0·20 1·0 H 0·50 1·0 0·10 0·50 1·0 0·05 1·0
Before
Citrated plasma
Activ. serum+citrate
Activated serum

FIG. 5.—Histamine release from a perfused guinea-pig lung, as assayed upon the guinea-pig gut after passage of citrated plasma incubated with agar for 30 min. at 38° (P_3 to P_8): activated serum with citrate added after incubation with agar (P_7 to P_{10}) and same serum without citrate (P_{11} to P_{15}). (H = histamine).

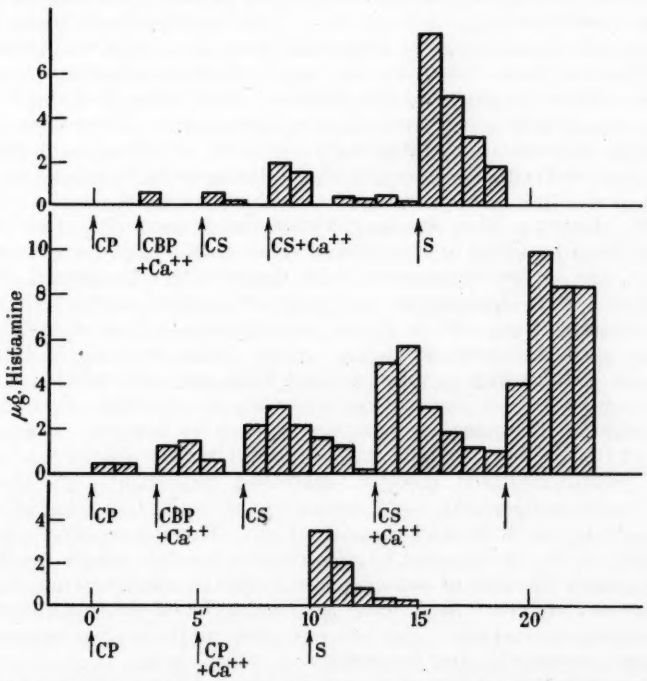

FIG. 6.—Histamine release from 3 guinea-pigs' lungs by perfusion with citrated plasma (CP), recalcified citrated plasma (CP + Ca^{++}), serum obtained by recalcification of citrated blood (CBP + Ca^{++}); citrated serum (CS); recalcified citrated serum (CS + Ca^{++}) and serum (S); all activated with agar for 1 hr. at 38°. Abscissae: time in minutes.

guinea-pig lung whether it is recalcified or not. If the $CaCl_2$ is added to the citrated blood and then the serum incubated with agar some very slight activation takes place. By using citrated serum more histamine was released than by using the corresponding citrated plasma. Recalcification of this citrated serum improved considerably its capacity of being activated by agar.

Interpretation of these experiments is not easy; it appears probable that the effect of agar needs an activating factor or co-factor that is given up by the cells when coagulation takes place. Citrate prevents the release of this factor completely and at least 90 per cent irreversibly when added to the total blood before clotting takes place. When added to serum, citrate reduces its capacity of being activated by agar but the effect is less strong than that on total blood. Some activator seems to escape the action of citrate after spontaneous clotting, although when the plasma is collected from citrated blood and then recalcified, or when recalcification is applied to total citrated blood, the effect of recalcification is slight or *nil*.

DISCUSSION.

The experiments presented in this paper show that normal blood contains a factor that can be activated to produce a conspicuous discharge of histamine from the perfused isolated guinea-pig lung. The relationship between the blood constituents and the discharge of histamine from dog's liver was postulated by Rocha e Silva and Graña (1946) on the basis that anticoagulants such as citrate and heparin reduced or abolished the release of histamine from dog's liver perfused with shock-producing agents such as peptone, ascaris extracts and so forth. Since platelets appeared to disintegrate in the liver and this disintegration was obviously connected with the intensity of the discharge of histamine and heparin from dog's liver either *in vivo* or *in vitro*, we have assumed (Rocha e Silva and Grãna, 1946; Rocha e Silva, Scroggie, Fidlar and Jaques, 1947) that the breakdown of platelets occurring in this kind of shock might play a major part in the discharge of the active substances from dog's liver. Dragstedt, Wells and Rocha e Silva (1942) showed that an excess of heparin partially inhibited the release of histamine from cells to plasma when samples of rabbit blood were put into contact with the shock-producing agents. More recently McIntire, Roth and Richards (1949), using samples of blood from sensitised rabbits, studied the shift of histamine from cells to plasma following the addition of the sensitising antigen. Addition of several anticoagulants, such as heparin, citrate, oxalate, large doses of $Ca^{\cdot\cdot}$ or $Mg^{\cdot\cdot}$, partially or totally inhibited the discharge of histamine. Soya bean trypsin inhibitor, however, decreased very slightly the discharge of histamine by the antigen but could completely stop the liberation of histamine by trypsin (McIntire, Roth and Sproull, 1950). The factors which participate in the clotting of the blood could be discarded as possible agents for the release of histamine, since the dose of anticoagulant to hinder the discharge of histamine was substantially different from those preventing blood-clotting (more heparin and less citrate or oxalate). Furthermore, the inhibition by citrate was 50 to 70 per cent irreversible after recalcification of the blood.

In the experiments on histamine release by anaphylatoxin we have seen a similar situation. When the blood is collected with 20 per cent of sodium citrate (3·8 per cent solution) the activation of anaphylatoxin by agar is completely

inhibited. Recalcification of the citrated blood or plasma results in no or very slight increase in its capacity to be activated by agar. If the citrate is added to the serum obtained by spontaneous clotting the process of activation is also considerably reduced, and recalcification definitely increased the effect of agar. According to the hypothesis that platelets might contribute with an activator or co-factor to the process of activation, one might explain this phenomenon by assuming that some intact platelets are still present in the serum and become protected by citrate and only partially disintegrate following recalcification. We hope to publish in the future experiments showing that heparin does not interfere with the activating process.

The mechanism by which serum becomes activated by treatment with agar may well be enzymic in nature. Heating the serum at 60° before the addition of agar completely destroys its capacity to be activated, as would be expected from work by Bordet (1913) on anaphylatoxin assayed on the intact guinea-pig, and by Novy and de Kruif (1917), who showed that at 70° only half of the anaphylatoxin activity was lost, but that heating at 100° completely destroyed its activity. We can assume therefore that the process depends on an activator that is destroyed at 60° for 1 hr., and that the active material (releasing factor) is somewhat more resistant to heating. As suggested by Novy and de Kruif, one might suppose that heat alters the precursor (matrix) of the anaphylatoxin in its molecular arrangement or denatures it.

In the earliest days of anaphylatoxin it became an accepted belief that the effect of agar, kaolin or barium sulphate on serum is to activate plasma protease by removal of the so-called anti-tryptic power of the blood. In a recent paper Bronfenbrenner (1944) discusses this idea on the basis of old and new experiments. There seems to be no doubt that in anaphylactic and peptone shock plasma protease is activated as indicated by the fibrinolysis that can be observed using the " protamine fibrinolytic test " (Rocha e Silva, Andrade and Teixeira, 1946). All attempts in our laboratory to show activation of a proteolytic enzyme after incubation with agar have been unsuccessful. Estimations of non-protein N or decreased viscosity of a gelatin solution entirely failed to show proteolytic activity in the serum activated with agar. Estimations of free α-amino-N in the serum submitted to incubation with agar have shown slight and possibly significant increases. However, the injection of a powerful preparation of fibrinolysin resulted in no release of histamine in lung perfusion experiments.

The histamine liberator—anaphylatoxin—released by incubation of normal serum with agar is probably not related to simple bases such as tubo-curarine, diamidines and so forth that are capable of releasing histamine (Alam, Anrep Barsoum, Talaat and Weininger, 1939; Rocha e Silva and Schild, 1949; MacIntosh and Paton, 1949; Reid, 1950), since the anaphylatoxin principle is destroyed by boiling and not extracted by hot alcohol. The complexity of the process of histamine release has often been overlooked. It is likely that several processes might be involved. Trypsin and other proteolytic enzymes displace histamine probably by hydrolysing a peptide linkage (Rocha e Silva, 1944); ammonia and simple tertiary or quaternary bases might release histamine by displacing it from its cell connections. Which mechanism underlies the releasing action of the histamine liberator released from serum by the action of agar and other polysaccharides is unknown. Its investigation might clarify the mechanism of liberation of histamine in anaphylaxis and inflammation.

SUMMARY.

Serotoxin (anaphylatoxin) prepared according to Bordet by incubating guinea-pig or ox serum with agar is capable of releasing considerable quantities of histamine when perfused through the isolated lung of the guinea-pig. The effect of temperature on the activation of serum has been studied.

Trisodium citrate added to whole blood before separation of the plasma, or to the serum after clotting of the blood, inhibited the formation of anaphylatoxin.

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PENICILLINASE ADAPTATION IN *BACILLUS CEREUS*: AN ANALYSIS OF THREE PHASES IN THE RESPONSE OF LOGARITHMICALLY GROWING CULTURES TO INDUCTION OF PENICILLINASE FORMATION BY PENICILLIN.

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THERE are many limitations to the interpretation of enzyme adaptation curves obtained when washed suspensions of cells are incubated in the presence of metabolizable substrate as inducer. Such conditions inevitably involve a varying concentration of substrate and thus a variable supply of possible building blocks and energy necessary for enzyme synthesis, as well as changes in other medium constituents and in rate of growth which may have non-specific effects on the adaptation process. Any interpretation of the kinetics of adaptation with such systems is thus very difficult. However, Monod (1950) has shown that it is possible, by the use of steady-state systems such as that obtainable by his technique of continuous culture, to maintain the concentration of inducer constant during continued growth and adaptation, as long as the inducer itself is not being metabolized by the enzyme during the process. The discovery (Monod, Cohen-Bazire and Cohn, 1951) that β-galactosidase formation by *Bact. coli* can be induced by galactose or melibiose in the absenec of detectable metabolism of either, enables this system to be used satisfactorily for the study of some aspects of the kinetics of adaptation (Monod, Pappenheimer and Cohen-Bazire, 1952). Penicillin-induced penicillinase formation in *Bacillus cereus* has similar properties, with the additional advantage that only transient contact with the inducer is necessary to enable cells to adapt fully to penicillinase formation, which can thus occur in the complete absence of free penicillin (Pollock, 1950). This peculiar and apparently unique property has been exploited, in the work described here, by following the effect of single additions of penicillin to cultures growing continuously in the logarithmic phase (*i.e.*, at maximal growth rate) during the whole course of the experiment. The low concentration of penicillin used—1·0 unit/ml. ($1{\cdot}7 \times 10^{-6}$ M) and below—has no effect on growth. All free penicillin in the medium can be destroyed within a few seconds by addition of an active preparation of penicillinase which also causes no interference with growth. This is a far gentler and more satisfactory way of removing all free penicillin than the washing procedure, previously used in the cold "pre-treatment" technique (Pollock, 1950).

It seems reasonable to assume that as long as exponential growth of cells persists, non-specific factors, such as limiting supply of nutrients and energy,

which might affect adaptation, are reduced to a minimum. The curve of penicillinase production so obtained was thus considered to be a more accurate reflection of the cells' specific response to penicillin than that achieved by other methods.

METHODS.

Medium.—Evans Peptone, 10 g., 3 g. Lab. Lemco and 2 g. NaCl were dissolved in 1 l. water. The pH was adjusted to 7·6 and the medium steamed for 30 min. and filtered to clarify. The pH was readjusted to 7·0 and the medium autoclaved at 10 lb. for 20 min.

Log-phase cultures were prepared by inoculating 50 or 100 ml. of the above medium with 1 drop of standard *B. cereus* NRRL 569 spore suspension (3×10^8 viable spores/ml.) followed by incubation on a shaker at 35°. The culture was grown for 3 to 5 hr. to the desired cell concentration (usually 0·05 to 0·2 mg. dry bacterial wt./ml.) and then gelatin (Difco) added to a final concentration of 1 per cent (to stabilize the enzyme), a little silicone DC Antifoam A smeared on the inside of the 250 ml. conical vessel employed and the culture transferred to an accurately controlled 35° water-bath and shaken at 100/min. with an amplitude of 5 cm. At this point 1 ml. of penicillin solution at the concentration required was added as rapidly as possible. In most experiments the penicillin was added when the culture had reached an opacity equivalent to 0·1 to 0·2 mg./ml. Opacity measurements were made every 5 or 10 min. by removing 0·5 ml. samples and estimating in the Spekker absorptiometer. The organisms continued growing exponentially in this medium until a cell concentration of about 0·8 mg./ml. was reached and experiments were never continued beyond this level.

Penicillinase activity of the combined extra- and intracellular fractions of the enzyme was measured manometrically after removing samples from the culture into M/1200 oxine and rapidly cooling to 0° to stop adaptation (Pollock, 1950), and expressed as μl. CO_2 liberated (by the penicilloic acid formed from the penicillin) from a bicarbonate buffer at pH 7·0 and 30° by 1 ml. of sample per hr. CO_2 retention in this medium was found to be negligible. The enzyme used for addition to cultures in order to destroy free penicillin was a highly active cell-free supernatant fluid (without any added preservative) prepared from a log-phase culture which had been allowed to grow for 2½–3 hr. after a single addition of 1 unit/ml. penicillin and then left in the ice-chest over-night. Such preparations had activities up to 10,000 μl./ml./hr.

Anaerobiosis was maintained, when desired, by transference of the culture to a 20 cm. × 3 cm. tube through which argon (< 10 vols. 0_2/million) was bubbled at 500 ml./min. *via* a Pasteur pipette. Aerobic conditions were restored, when necessary, by switching from argon to air at the same rate for 60 sec. and then transference back to aerobic shaking in a 250 ml. flask as before.

Penicillin used was the Na salt of benzyl penicillin (Glaxo) containing 1670 international units/mg. Radioactive ^{35}S-labelled benzyl penicillin with specific activity of about 0·18 μc/unit was prepared by C. J. Perret (Pollock and Perret, 1953), and the fixation on the cells of ^{35}S from this penicillin followed by the technique used previously (Pollock and Perret, 1951). Maximum non-penicillin S impurity present was 10–15 per cent, most of which was almost certainly penicilloic acid.

RESULTS.

Fig. 1 shows the rates of penicillinase formation by two logarithmically growing aerobic cultures of *B. cereus*, to one of which (A) 1 unit/ml. penicillin was added only a few minutes after their origin from a common culture. Excess enzyme (final concentration of 250 μl./ml./hr.) was added to both 60 sec. after addition of penicillin. Such added enzyme was shown to remain stable under these conditions for at least 3 hr., and forms a sufficiently small proportion of the adaptively produced enzyme to avoid obscuring the results. If this amount of enzyme is mixed with the penicillin only 10 sec. before adding both to the culture, no adapta-

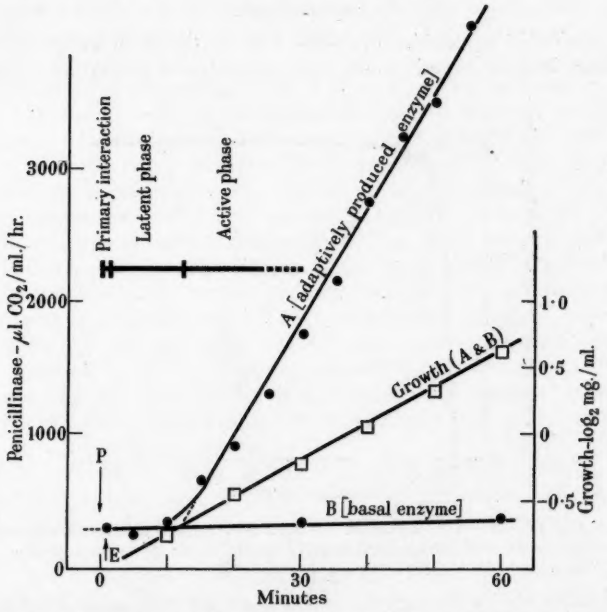

FIG. 1.—Formation of penicillinase by logarithmically growing cultures of *B. cereus* in 1 per cent gelatin broth, showing the 3 phases of adaptation. A, after addition of 1 unit/ml. penicillin; B, control culture without penicillin.

P = penicillin added to A.
E = penicillinase (250 μl./ml./hr.) added to A and B to destroy all free penicillin.

tion whatever occurs. Thus it could be concluded with assurance that all of the remaining free penicillin was destroyed by the added enzyme almost instantaneously. Growth is unaffected by the addition of penicillin and continues exponentially for the whole 60 min. of the experiment. The so-called "basal" enzyme formed by the control culture is relatively very low, showing a slight, barely significant increase during this period.

The form of the curve obtained under the conditions of this experiment seemed to justify its subdivision into the three following phases: (1) "Primary Interaction" between penicillin and cells lasting up to the time when all free

penicillin is destroyed—which, in the above experiment, is determined by the time of addition of penicillinase; (2) a "Latent Phase" lasting about 15 min. between the end of phase (1) and the time when a constant rate of penicillinase formation is attained; and (3) an "Active Phase" during which penicillinase is produced at approximately constant rate apparently indefinitely as long as the cells continue their logarithmic growth. As previously pointed out (Pollock, 1950), this of course entails a gradual decrease in the amount of penicillinase per unit cell mass so that the original basal (unadapted) level is reached, for all intents and purposes, after about 10 cell generations.

Active Phase.

Effect of penicillin concentration.—The rate of penicillinase production during the active phase is determined by the concentration of penicillin originally added,

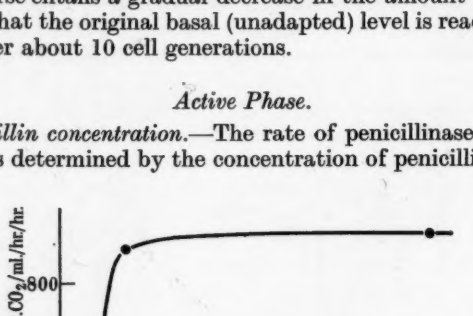

FIG. 2.—Relation between concentration of penicillin added to logarithmically growing cultures of *B. cereus* and rate of subsequent penicillinase formation during the linear ("active") phase.

in exactly the same relationship as that found with an aerobically incubated suspension pre-treated with penicillin at 0° for 1 hr. (Pollock, 1950). The curve of relationship is hyperbolic with a maximum at about 1·0 unit/ml. and "half-maximum" at about 0·2 unit/ml. (3×10^{-7} M) (Fig. 2). Between the range of 0·01 and 0·1 unit/ml. the relationship is approximately linear (Fig. 3*a* and *b*).

Effect of O_2 withdrawal.—In order to distinguish between the possibly different types of metabolism underlying the three phases, a search was made for a suitable reversible inhibitor. It soon became clear that O_2 itself was a very useful tool since it can be rapidly removed or added at will. Substitution of argon for air during the active phase causes an instantaneous inhibition of both growth and enzyme production; and restoration of air after 25 min. anaerobiosis allows immediate resumption of both growth and penicillinase formation at the same rate as before (Fig. 4). The effect of O_2-lack at this stage will be dealt with in detail in a later publication.

The failure of enzyme formation in the absence of O_2 is not in itself surprising.

The important feature is the immediate cessation and restoration of both growth and penicillinase formation without any appreciable intermediate stages of " tailing off," or of lag on recovery.

Latent Phase.

The transition from the period of no enzyme formation, after addition of penicillin, to that of maximal enzyme formation is not instantaneous. There is an intermediate period of rapidly increasing rate of enzyme production. In order, therefore, to obtain significant and reproducible figures for the length of lag, it has been measured as the interval between the end of phase (1) (*i.e.*, time of addition of enzyme to destroy free penicillin) and the intersection of the extrapolated line of the slope of the active phase with a line parallel to the abscissa drawn through the estimated enzyme value at 1 min. Determined in this way, the mean duration of the latent phase is 14 (S.D. $\pm$ 1·7) min. based on 19 different estimations. It should be emphasized that this latent phase is specific to penicillinase formation—that is, there is no corresponding lag in growth, which continues exponentially over the whole period of the experiment. It is thus quite different from the much longer lag in enzyme production usually shown by washed suspensions during adaptation, which is probably largely determined by the same factors as those responsible for the ordinary lag in growth shown by all cells cropped after the end of the logarithmic phase when placed in a fresh medium.

Effects of penicillin concentration and cell concentration.—Neither variations in the concentration of penicillin from 0·1 to 1·0 unit/ml. added in stage (1) at a cell concentration of 0·1 mg./ml. (Fig. 5) nor variations in cell concentration from 0·05 to 0·5 mg./ml. with an inducing penicillin concentration of 1·0 unit/ml. (Fig. 6) caused any significant difference in length of the latent phase, although there were 4-fold and 10-fold differences respectively in the rate of enzyme production during the active phase.

Effect of O_2 withdrawal.—Fig. 7*a* and *b* show that after both 12 and 25 min. anaerobiosis, there is the same latent phase of approximately 15 min. before the active phase begins. It has already been shown that restoration of O_2 after anaerobiosis during the active phase allows immediate resumption of enzyme formation. Thus if the processes underlying the latent phase had been able to occur normally during anaerobic incubation, enzyme formation should have begun immediately on restoration of air. But, as far as can be judged, even 25 min. anaerobic incubation did not contribute towards a shortening of the subsequent lag.

Primary Interaction.

The initial reaction between penicillin and cells as it takes place in growing cultures at 35° clearly corresponds closely with the reaction occurring during pretreatment of washed cells with penicillin at 0°. There are, however, some important differences. At 0° the small traces of enzyme present in the cells after washing must hardly function at all and the added penicillin remains almost entirely undestroyed (Pollock and Perret, 1951). Under these conditions the reaction between penicillin and cells ceases through the attainment of some sort of equilibrium, since even at penicillin concentrations well below that producing maximum adaptation and maximum specific fixation of penicillin sulphur on the cells, only a very small fraction (20 per cent) of penicillin actually disappears and

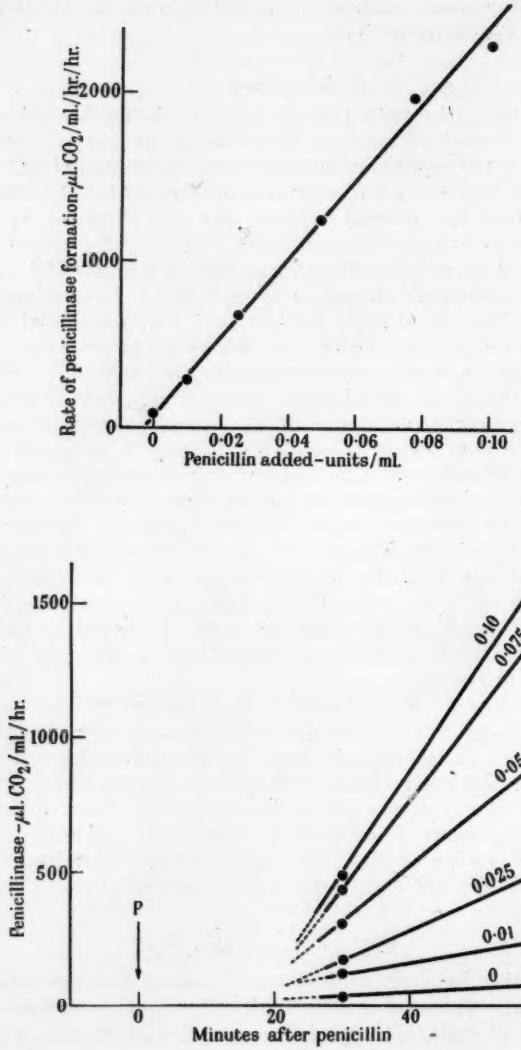

FIG. 3.—Relation between lower concentrations of penicillin added to logarithmically growing cultures of *B. cereus* and rate of subsequent penicillinase formation.

(*a*) Penicillinase formation curves in comparable cultures to which different penicillin concentrations (indicated on curves in units/ml.) were added at P.

(*b*) Rates of penicillinase formation during active phase (*i.e.*, differences between 60 min. and 30 min. values from (*a*)) plotted against concentration of penicillin added.

an even smaller fraction (0·2 per cent) of penicillin S is fixed on the cells. At 35°, with growing cultures, on the other hand, the primary interaction between penicillin and cells is rapidly brought to an end by enzymic destruction of the penicillin. Although in most of the experiments here described this phase is ended by "artificial" addition of penicillinase, it can easily be shown that the penicillin would in any case be rapidly destroyed by the basal enzyme that is already present in the culture—and, at 35°, fully functional. Addition of enzyme more than 5 min. after addition of concentrations of penicillin below 10 units/ml. does not in fact make much difference to the subsequent rate of enzyme formation, and is only done in order to sharpen and fix accurately the end of the phase of primary interaction. In fact, in the experiments concerned only with the active phase (see Fig. 3 and 4), this enzyme addition 1 min. after penicillin has been omitted.

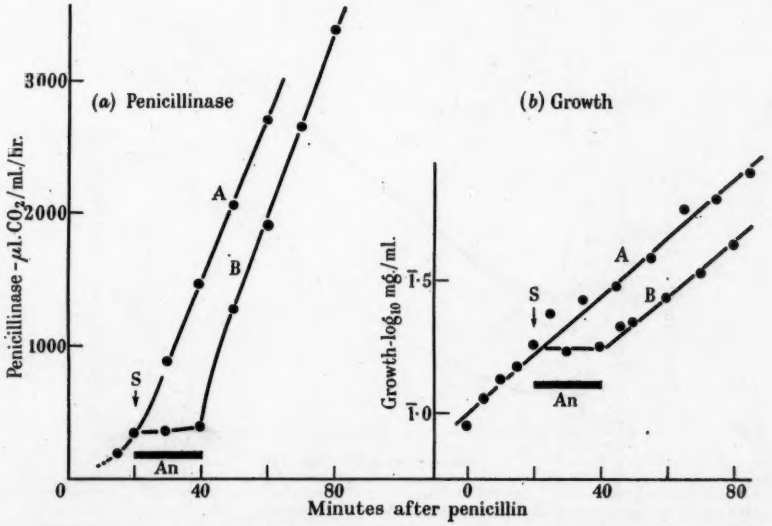

FIG. 4.—Effect of 20 min. anaerobiosis on (*a*) adaptive penicillinase formation, and (*b*) growth, of *B. cereus* cultures growing logarithmically in 1 per cent gelatin broth. A, control culture growing aerobically all the time. B, culture with anaerobic conditions (applied by bubbling argon through at 500 ml./min.) for 20 min. as indicated by broad horizontal line (An).

Penicillin (1 unit/ml.) was added to the communal culture at 0 min. before dividing into A and B at s, 20 min. later.

Velocity.—Even at 0°, the reaction was found to proceed rapidly, being 50 per cent complete in about 10 min. At 35°, with growing cultures, it was followed by adding enzyme to destroy free penicillin at different intervals after addition of penicillin and comparing the rates of subsequent penicillinase formation. Fig. 8 shows the result of such an experiment in which ^{35}S-labelled penicillin was used and the speed of uptake of penicillin S compared directly with the speed of interaction between penicillin and cells to induce penicillinase formation. The technique was as follows: six 100-ml. log-phase cultures were pooled and redistri-

buted in 6 lots. Radioactive penicillin to a final conc. of 0·1 unit/ml. was added to 5 lots as rapidly as possible, followed by penicillinase (final concentration of 250 μl./ml./hr.) after 10 sec., 30 sec., 60 sec., 2 min. and 5 min. respectively, during continued aerobic incubation. To the remaining lot was added a mixture of penicillin and enzyme previously incubated together for 10 sec. at 35° before addition ("zero-time" culture). After 5 min., 10 ml. was retained from each culture for continued aerobic incubation and penicillinase production. while the remaining 90 ml. were centrifuged at 0° as rapidly as possible, the cells washed 3 times and the amount of S fixed on the cells measured by the method described. The amount of S fixed on the cells from penicilloic acid at a concentration of 0·1 unit-equiv./ml. is negligible—as shown by the "zero-min." value; so that it can be safely assumed that all S fixed by the cells in this experiment is specifically from penicillin. The results show that primary interaction at 35° is

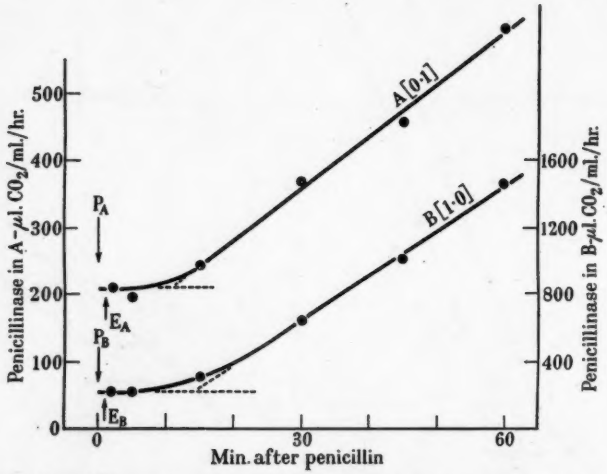

FIG. 5.—Effect of penicillin concentration on length of the "latent phase" in penicillinase adaptation of logarithmically growing cultures of *B. cereus*.

P_A, P_B, addition of 0·1 and 1·0 unit/ml. penicillin to identical cultures A and B respectively.

E_A, E_B, addition of penicillinase (250 μl./ml./hr.) to A and B respectively, to destroy free penicillin.

extremely rapid. Postponement of addition of enzyme for 30 min. did not allow much more rapid penicillinase formation than that occurring after enzyme addition at 5 min. Thus, within the limits of the technique, primary interaction is normally over within about 5 min., and is 50 per cent complete in approximately 60 sec. The close relation between the rates of fixation of penicillin S on the cells and that of the "primary interaction" phase in penicillinase adaptation is obvious.

Effect of O_2 withdrawal.—Fig. 9 shows that if addition of penicillin, and enzyme 60 sec. later, is made just before the end of 25 min. anaerobiosis, the subsequent latent and active phases are unaffected. Thus even though the whole 60 sec. period of primary interaction takes place under anaerobic conditions, the cell response to penicillin after restoration of air is in no way altered.

Effect of penicillin concentration.—This has not been studied in detail with growing cultures at 35°, although it is known that the speed of the pre-treatment effect at 0° is unaffected by the concentration of penicillin (Pollock and Perret, 1951).

DISCUSSION.

The phase of primary interaction is closely paralleled by specific uptake of S from penicillin; it appears not to be dependent on cell metabolism, since it occurs rapidly at 0° and is not affected by withdrawal of O_2. It behaves more as if it were a chemical or physio-chemical reaction involving penicillin and some specific cell receptor, with the formation of a new complex or compound:

$$\text{Penicillin} + \text{Receptor} \rightleftharpoons \underset{(PR)}{\text{Penicillin-Receptor Complex}}$$

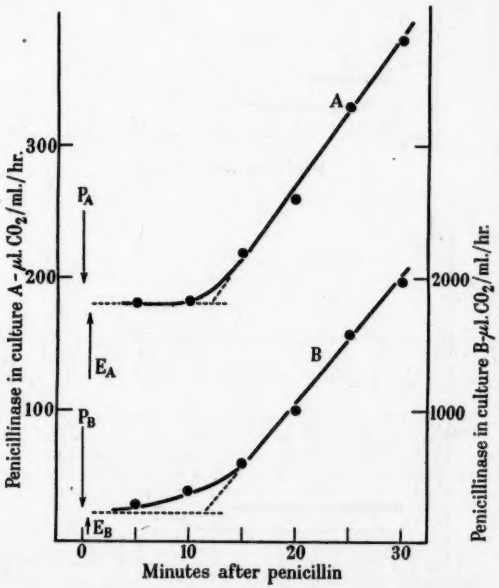

FIG. 6.—Length of the "latent phase" in penicillinase adaptation following addition of 1 unit/ml. penicillin at different cell concentrations to logarithmically growing cultures of *B. cereus*.

Culture A: Penicillin added (P_A) when cell conc. reached 0·05 mg./ml.
Culture B: Penicillin added (P_B) when cell conc. reached 0·50 mg./ml.

E_A, E_B, addition of penicillinase (250 µl./ml./hr.) to A and B respectively, to destroy free penicillin.

The evidence supporting the hypothesis that the "PR" complex acts directly or indirectly as some sort of specific catalyst, whose concentration determines the rate of penicillinase formation, has already been outlined and considered in previous papers. On the whole, it is thought that results reported here lend further support to this idea. The most probable basis for the primary interaction phase

is the reaction between penicillin and some cell receptor which involves the firm binding of penicillin S.

Even apart from the results with ^{35}S-labelled penicillin, it follows that after addition of the penicillinase no free penicillin can be present; if still in existence

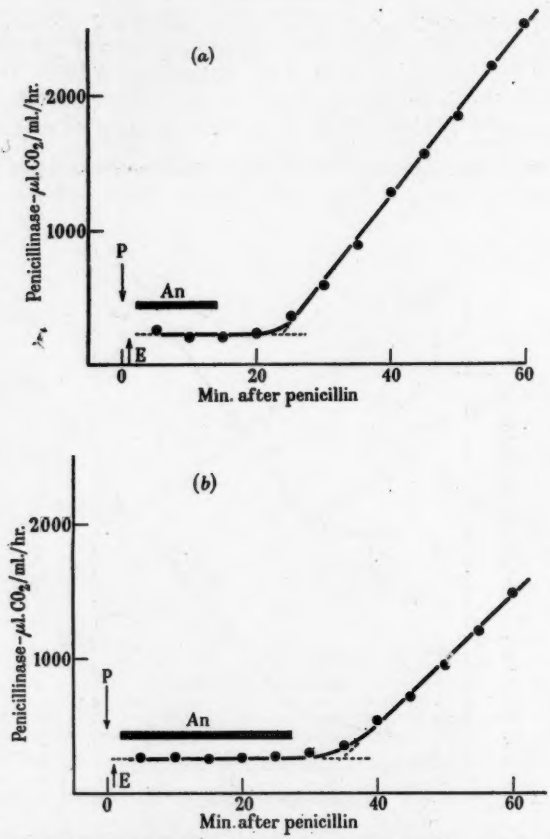

FIG. 7.—Effect of anaerobic conditions, applied immediately after addition of penicillin to logarithmically growing cultures of *B. cereus*, on subsequent penicillinase production, showing persistence of "latent phase" following restoration of air.

P, addition of 1 unit/ml. penicillin.
E, addition of penicillinase (250 μl./ml./hr.) to destroy free penicillin.

Duration of anaerobiosis (argon bubbling through cultures at 500 ml./min.) is indicated by broad horizontal line (An) lasting for 12 min. and 25 min. in (*a*) and (*b*) respectively.

it must be fixed in or on the cell in some way which prevents it from being attacked by the enzyme and from diffusing freely out from the cell. It is difficult to avoid the conclusion that it is adsorbed on, or has chemically reacted with, some cell component.

The " Active Phase " needs little comment at the moment. It corresponds to the biosynthesis of active enzyme, at a rate dependent on the concentration of penicillin added, is closely bound with the metabolism of the growing cell, and must include the last stage in the complicated chain of reactions involved in penicillinase adaptation. It may, in fact, include all the stages in the production of penicillinase beyond the formation of the hypothetical PR or active catalyst.

The " Latent Phase " is the most interesting and the most difficult to interpret. Is it really anything more than a transition between primary interaction and active enzyme formation ? In other words, are the processes controlling it fundamentally different from those underlying the active phase ? If they are not different, it is necessary to explain why enzyme formation does not begin at maximal rate immediately after the first phase is complete ; for the cells are

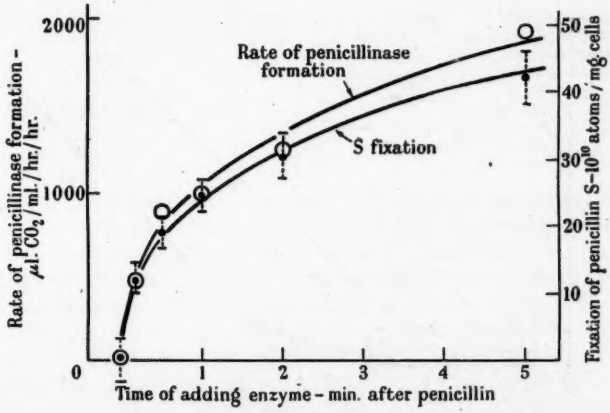

FIG. 8.—Velocity of " primary interaction " phase. Rate of subsequent penicillinase formation and specific fixation of ^{35}S from penicillin on the cells after addition of 0·1 unit/ml. of ^{35}S-labelled penicillin to logarithmically growing cultures of *B. cereus* followed by penicillinase (to destroy all free penicillin) at different intervals in different cultures. For details see text. Limits of counting error shown for P = ·05.

growing at maximal rate and have no corresponding lag. An obvious possible explanation would be the existence of some specific enzyme precursor whose formation might begin at maximal rate immediately after primary interaction, but would not lead to production of active enzyme at maximal rate until the precursor concentration had reached a level such that its transformation into active enzyme equalled the rate of its own production. The time taken to reach this point might of course depend upon a number of factors, such as the relationship between precursor concentration and rate of enzyme formation from precursor, which at the moment are not measurable. But as long as the rate of active enzyme production from precursor was not extremely rapid compared to that of precursor formation, some lag in enzyme formation must occur.

The other main alternative interpretation of the latent phase is that it represents some specific readjustment of cell metabolism corresponding to the " métabolisme des inducteurs " postulated by Monod *et al.* (1951). The hypothetical

" PR " complex might, for instance, be further metabolized to another specific compound which acts as the true enzyme-forming catalyst ; or it might be incorporated into a more complex structure, such as a catalytic metabolic system analogous to the Krebs cycle.

Whatever the explanation of the latent phase may be, it is clear that the processes underlying it are dependent upon a source of energy—*i.e.*, they are intimately bound up with the cell's general metabolism—since they are inhibited by lack of O_2. This, by itself, makes it very unlikely that the latent phase is due mainly to some physio-chemical process such as internal diffusion of the bound penicillin or active catalyst. It must be, essentially, a metabolic process —and one, in fact, which involves changes which are exclusively intracellular. Fig. 6 shows that the length of the phase is not affected by a 10-fold variation in cell concentration. If it were affected by an accumulation of extracellular

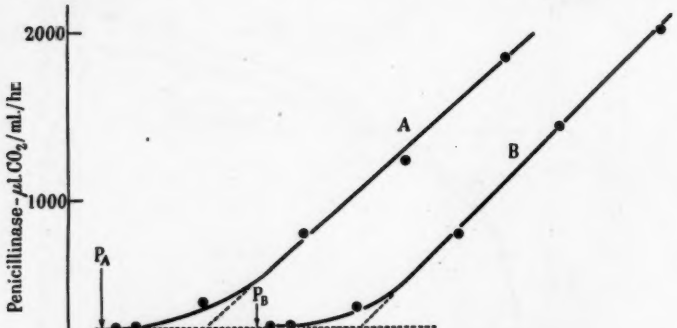

FIG. 9.—Effect of anaerobic conditions applied during the phase of primary interaction between penicillin and cells of a logarithmically growing culture of *B. cereus*, on subsequent formation of penicillinase.

P_A P_B, addition of 1 unit/ml. penicillin to aerobic (control) culture (A) and anaerobic culture (B) respectively.

E_A, E_B, addition of penicillinase 250 μl./ml./hr. to A and B respectively.

Broad horizontal band (An) indicates period of anaerobiosis (argon bubbled through at 500 ml./min.) applied to B.

metabolites formed by the cells themselves its duration would be expected to vary with the cell concentration (as indeed is the case with many forms of lag in the growth of organisms freshly inoculated into a medium). It is thus reasonable to conclude that the latent phase is the reflection of a specific intracellular metabolic process. Further attempts at characterization would be pure speculation. It might be one or a number of processes, and it is quite possible that the formation of some specific enzyme precursor plays a part. Its importance lies in the recognition that it is a feature worth studying as a distinct phenomenon.

In order to avoid possible confusion later on it is suggested that cells which have completed the phase of primary interaction be referred to as " penicillin-primed " ; and those that have passed through the latent phase be termed

"penicillinase-competent." Penicillinase adaptation would then still refer to the whole process, including all three phases. A summary of these three phases, with a tentative analysis and interpretation, is given in the Table.

TABLE.—*Stages in Penicillinase Adaptation* (B. cereus) *at* 35°.

Phase.	Characteristics.	Term applied to cells when phase is complete.	Interpretation.
Primary interaction	Very rapid (about 50 per cent complete in 60 sec.). No energy required	"Penicillin-primed"	Probably simple chemical reaction or physico-chemical adsorption, *e.g.*, Penicillin + Receptor $\rightleftharpoons$ Receptor-Penicillin complexor compound (PR).
Latent	Energy-requiring (*i.e.*, linked with cell metabolism). Duration: approx. 15 min.	"Penicillinase-competent"	? Further metabolism of PR to active compound. ? Formation of inactive enzyme precursor.
Active	Energy-requiring	—	Formation of specific active enzyme protein—at rate proportional to [PR].

As previously mentioned, it is uncertain yet whether penicillinase adaptation is unique in that increased enzyme production does not require the continued presence of free inducer as with other enzyme adaptations. If the basic stages are the same in all enzyme adaptations, then in other cases one of the early steps must be so unstable that primary interaction must be continuous for enzyme formation to persist. Thus, during active enzyme formation, both primary interaction and the processes underlying the latent phase would be occurring all the time. It would clearly be difficult to separate the different phases one from another. On the reasonable assumption that the fundamental basis of penicillinase adaptation is the same as for other enzyme adaptations, it is hoped that further analysis and characterization of the three phases here described will help in the understanding of the mechanism of other enzyme adaptations in micro-organisms.

SUMMARY.

Penicillinase production in the absence of free penicillin has been followed after addition of penicillin to logarithmically growing cultures of *B. cereus*, and subsequent destruction of free penicillin by a penicillinase preparation.

Growth of the cultures is unaffected by addition of penicillin and enzyme, so that there is likely to be minimum variation in non-specific factors affecting penicillinase formation, and interpretation of the kinetics is thus simplified.

By these means it has been possible to divide the phenomenon of penicillinase adaptation into three phases:

1. A phase of *Primary Interaction* between penicillin and cells, 50 per cent complete in 60 sec. at 35°, involving the specific fixation of penicillin S on some cell receptor with the formation of a new compound or complex. It appears to be largely independent of cell metabolism.

2. A *Latent Phase*, during which little enzyme is formed, lasting about 15 min. It is unaffected by variations in penicillin concentration or cell numbers

but is a necessary preliminary to the third phase. The metabolic processes underlying it are inhibited by lack of O_2.

3. An *Active Phase*, during which total penicillinase in the culture is formed at a constant rate determined by the concentration of added penicillin up to a level of 1·0 unit/ml. above which no further increase occurs. It is immediately inhibited by anaerobic conditions, but restarts without lag at the original rate on restoration of O_2.

I wish to thank Mr. C. J. Perret for preparing the purified ^{35}S-labelled benzylpenicillin used in this work; and I am most grateful to Miss Daphne Towler for expert technical assistance.

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A COMPARISON BETWEEN INTRANASAL INSTILLATION AND NATURAL INHALATION AS MEANS OF INFECTING MICE WITH INFLUENZA VIRUS.

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In the course of quantitative work with influenza virus we performed on several occasions 5 to 6 replicate titrations within a single day of a 10 per cent suspension of virus; dilutions spaced at half-log intervals were instilled intranasally under ether anaesthesia into groups each of six mice. Statistical analysis of the results, expressed conventionally as the pulmonary scores on the seventh day or the weighted score where death had occurred earlier, showed that the variations between mice receiving the same dilutions were consistent either with wide differences in susceptibility of individual animals or with wide variations in the amount of virus reaching the lungs. Assuming that all the variations were due to the second factor, in one representative set of titrations they were such that for dilutions of virus between 10^{-1} and $10^{-3.5}$ the doses of virus might have ranged from 1 to 10,000 in different mice, while for dilutions higher than this the doses might have ranged from 1 to 900. Other titrations yielded results with even greater irregularities. While it seemed unlikely that properly anaesthetised mice apparently inspiring satisfactorily the drops of viral suspension in fact received grossly variable amounts of virus, we put the matter to the test by instilling in place of virus a 1 per cent solution of Lissamine green V or other suitable dye. Autopsy five minutes later disclosed the widest variations in the amount of dye reaching the lungs; some lungs obviously contained a fair proportion of the dye administered, others little or none. In one experiment our colleague Mr. J. M. Thorp kindly estimated the amount recoverable; it varied from about 15 per cent of that administered to quantities so small as to be undetectable colorimetrically. Moreover, when dye was present in the lung its distribution was grossly uneven in the different parts. This unexpected result was duplicated by a very experienced worker in another laboratory.

It seemed at least possible that where large numbers of mice were to be infected at one time with a single dilution of virus less variable results might follow its administration by some method other than instillation. Many workers, of course, have infected mice or other animals by natural inhalation of virus-containing mists (Wells and Henle, 1941; Edward, Elford and Laidlaw, 1943; Loosli, Robertson and Puck, 1943; Krueger *et al.*, 1944; Stone and Burnet, 1945; Bourdillon and Glover, 1948*b*), while the problems of experimental air-borne infections in general have been studied by means of apparatus varying greatly in type and complexity (Twort, Baker, Finn and Powell, 1940; Baker, Finn and

Twort, 1940; Wells, 1940; Wells and Lurie, 1941; Loosli, 1944; DeOme, 1944; Faber, Silverberg and Dong, 1944; Rosebury, 1947; Bourdillon and Glover, 1948*a*; Sonkin, 1951; Weiss and Segeler, 1952; and others). Edward, Elford and Laidlaw suggested that in large batches of mice inhalation might lead to more uniform influenzal infection than can be obtained by intranasal instillation of virus, and Glover (1944) showed this to be the case with tubercle bacilli. Krueger *et al.* (1944), using india ink or radio-active chromic phosphate in monkeys, emphasized the superiority of inhalation over nasal instillation, which in their words "provides a rather direct route to the alimentary tract." Loosli, Robertson and Puck also compared inhalation and intranasal instillation as methods of infecting mice with influenza, although they were not primarily concerned with whether or not more uniform infections resulted from the former. In fact, as far as influenza is concerned we are aware of no precise comparison of the relative merits of the two methods, and it is the purpose of this report to supply such information.

APPARATUS AND METHODS.

Apparatus.

The apparatus we have used is simple in the extreme, yet is capable over a period of giving reproducible results. We should, of course, repeat that our object has been to infect simultaneously and as uniformly as possible large numbers of mice with a virus of relatively low pathogenicity for man, rather than to study the variables of respiratory infection with maybe highly dangerous organisms. A well-made painted wooden cabinet approximately 4 ft. × 3 ft. × 2 ft. 6 in. (122 × 91 × 76 cm.) in external dimensions is fitted at the front with a door 18 in. square screwing down by butterfly-nuts onto a rubber gasket; this door could with advantage be larger. One side of the cabinet consists partly of a large air-tight observation window measuring 2 ft. 9 in. × 1 ft. 9 in. Inside on each side of the cabinet are three "open" shelves made of angle-iron, each capable of housing five half-inch wire-mesh boxes measuring 6 in. × 6 in. × 3½ in.; these boxes conveniently hold up to five or more mice. The place of one box is reserved for a humidifying bottle, and of another on the opposite side of the cabinet for a wet-dry-bulb thermometer. The lowest shelf is 12 in. from the floor of the cabinet and the highest 20 in. from the ceiling. Centrally on the floor of the cabinet is a 6-in. three-bladed fan rotated by a fractional horse-power motor at 2000 r.p.m.; it is so wired that it will run only when the door is closed. As measured with a hot-wire anemometer, the upward air velocity immediately above the fan is 5 ft./sec., while 3 in. away from the walls of the cabinet the downward velocity is 1·5 ft./sec. On the ceiling and on the lowest part of the sides of the cabinet are four Hanovia SNJ 10-in. jacketed ultra-violet tubes. Apertures at the top front and bottom rear are closed with tightly fitting rubber bungs carrying tubes for the entry of compressed air or the escape of air through two fairly loosely packed cotton-wool filters placed in series; the distal filter is impregnated with iodine (see Stone and Burnet, 1945), and is in position only while air is passing through the box. The laboratory supply of compressed air is relayed through a needle-valve and a storage cylinder fitted with a pressure gauge and safety valve and enters a nebulizer at a pressure of 10 lb./sq. in. No positive pressure develops in the cabinet during operations. The nebulizer, an ordinary commercial article of the all-glass aspiratory type ("Dritex"—Messrs. Riddell Products, Ltd.), is so placed 12 in. from the ceiling at the front of the cabinet as to direct its mist upwards at an angle of about 40° from the horizontal; although the instrument is visibly not a standard article, it has the advantage of being readily replaced if broken.

Method of use.

Fifteen to 30 min. before the start of an experiment the mice receive orally 0·25 mg./20 g. amphetamine sulphate, which induces them to move continuously during the experiment instead of huddling together in a corner of the cage. They are then placed in the cabinet. Four ml. of a suitable virus suspension in 10 per cent horse-serum saline are introduced

into the nebulizer and the humidifying bottle filled with water at about 70°. The fan is started. Compressed air is bubbled via an " Aerox " filter through the initially hot water until the wet-bulb thermometer attains a temperature not more than 0·6°(1°F.) lower than the dry-bulb thermometer (96 per cent humidity). Air at 10 lb. pressure is next directed through the nebulizer for 1 hr., a period more than sufficient to dispense all the viral suspension (see later). Humidity in the cabinet is now 96–98 per cent and the temperature, initially 21–29°, has risen by 0·5–2°. With the fan still running the ultraviolet lamps are turned on for ½ hr.; during this period the supply of air through the humidifying bottle is turned off and is replaced by dry compressed air. The mice are then transferred to their permanent cages. As a matter of interest, at the end of operations we have failed to recover influenza virus from the coats of 50 mice washed in the smallest feasible amount of saline solution.

Remarks on conditions prevailing within the cabinet.

It will be noted that in contrast to almost all other workers we have performed our experiments under conditions of very high relative humidity. Both with bacteria and with viruses most authors have claimed that high humidity is inimical to survival. Thus Edward, Elford and Laidlaw (1943) found that infectivity persisted longer in a dry than in a wet aerosol of influenza virus, and thought that the droplets of the latter might settle more quickly. Loosli, Lemon, Robertson and Appel (1943) confirmed this observation, but attributed the disappearance of infectivity to actual inactivation of virus rather than to settling of the particles. DeOme (1944) showed that the death-rate of *Salm. pullorum* dispensed from water into a dust-free atmosphere increased with rising humidity, though Rosebury (1947) obtained in general better recoveries of two bacteria and a virus under conditions of high humidity. However this may be, when initially we sprayed virus into an atmosphere of about 40 per cent relative humidity we obtained inferior and much more variable results than under the experimental conditions described above. Our conditions and requirements differed in several material respects from those commonly described in the literature. Thus wishing to obtain immediate infection rather than to study survival of the virus, we generated the aerosol within the cabinet in close proximity to the animals to be infected and kept the air in a state of vigorous circulation tending to minimize settling of droplets. It has been shown that particles of a certain size are optimal for retention in the lower respiratory tract; above this size they stick in the upper respiratory passages, substantially below it they are less completely retained in the lungs (see van Wijk and Patterson, 1940). The optimal size is given as from 0·7 to 3μ (according to the test-substance) by Landahl and Herrman (1948), 1μ for china-clay dust by Brown, Cook, Ney and Hatch (1950), and 1·8μ for droplets containing haemolytic streptococci by Sonkin (1951). The makers of the nebulizers we have used in these experiments claim that the range of particle-sizes emitted varies from 3–20μ. Mr. H. A. Druett of the Microbiological Research Station, Porton, very kindly measured the particle sizes of 10 per cent horse-serum saline emitted into *dried* air by these nebulizers. Over 90 per cent of the particles were below 0·8μ and approximately 40 per cent were below 0·3μ in diameter. It seems that if droplets emitted from our nebulizers were allowed to desiccate they might be less easily retained in the lungs than if discharged into an atmosphere nearly saturated with water vapour.

Our experience suggests that, provided that the virus-containing mist is produced in close proximity to the animals to be infected and settling is minimized by vigorous circulation of air, the high relative humidity, far from being deleterious, is actually an advantage in the type of work under consideration.

RESULTS.

Comparison of Nasal Instillation with Natural Inhalation as a Method of Infection with Influenza Virus.

We carried out four experiments over a period of approximately three months, during which time the virus lost some of its initial activity. The mice (20 g. ± 1 g.) were divided into four groups of fully randomised animals, of which two groups chosen at random were used in the morning and two in the afternoon.

One group in the morning was subdivided into small groups each receiving one of two or three dilutions of virus (PR8 strain of influenza A virus) by intranasal instillation. The other group was subdivided into 24 sub-groups identified by colour and infected in different recorded positions in the inhalation cabinet. The afternoon operation was similar except that a different dilution of virus from that in the morning was used for inhalation. In all cases dilutions of virus were made with 10 per cent horse-serum saline from an initial centrifuged 10 per cent suspension of infected mouse-lung in horse-serum saline. On the 7th day surviving mice were killed and scored in the usual way according to the degree of pulmonary consolidation. Mice dying specifically before this received the scores 8, 7, 6 or 5 according to whether they died on the 4th, 5th, 6th or 7th days. Table I summarises the results. Since the mean scores obtained by the two methods of infection did not tally as exactly as we had tried to ensure, and since from Table I it is apparent that variability between mice is related to the mean score,

TABLE I.—*Comparison of Mice Infected with Influenza by Intranasal Instillation and by Inhalation.*

	Intranasal instillation.				Inhalation.			
Experiment.	Dilution of virus.	Number of mice.	Mean pulmonary score.	Variance within groups.	Dilution of virus.	Number of mice.	Mean pulmonary score.	Variance within groups.
1	$10^{-5.5}$	48	4·73	4·22	$10^{-2.0}$	72	5·07	2·26
	$10^{-6.0}$	48	2·50	2·21	$10^{-3.0}$	72	2·97	1·14
	$10^{-6.5}$	48	2·11	1·49				
2	$10^{-5.0}$	72	5·53	2·89	$10^{-2.0}$	72	4·60	2·33
	$10^{-6.0}$	72	3·57	2·90	$10^{-3.0}$	72	2·31	0·94
3	$10^{-5.5}$	72	3·52	2·55	$10^{-2.0}$	72	3·88	2·07
	$10^{-6.5}$	72	1·80	0·92	$10^{-3.0}$	72	1·85	0·85
4	$10^{-5.0}$	72	4·17	2·31	$10^{-1.7}$	72	4·43	2·56
	$10^{-6.0}$	72	2·00	1·36	$10^{-2.7}$	72	2·89	1·78

we also compare the two sets of data in the Figure. From both table and chart it is clear that in 3 of 4 experiments the inhalation method gave results much less variable than those obtained by intranasal instillation. In the fourth experiment variability with intranasal instillation was appreciably lower than usual for the more concentrated virus, while with inhalation it was higher than usual for the lower concentration; from this experiment alone no difference in variability between the two methods could be claimed, but on the basis of all four experiments there can be no doubt that inhalation gives the more uniform results. In many subsequent experiments using smaller groups of animals, mouse-to-mouse variation has always been between the limits noted above.

In the inhalation experiments we paid special attention to the possibility of variations arising from the different positions of mice within the cabinet. Although in individual experiments a particular situation might be associated with high or low pulmonary scores, we observed no consistent tendency for position in any one part of the cabinet to lead to appreciably different results; a complete analysis of the variance of the results was statistically consistent with the conclusion that virus was uniformly distributed.

Experiments with Bacteria.

The foregoing data show that variation between mice can be materially reduced by substituting inhalation for intranasal instillation when infecting them with influenza virus. Since, however, they record the pulmonary scores 7 days after exposure to virus rather than the amount of virus present in the lungs immediately after contact, they do not show how far the variation still persisting is inherent in the technique of infection and how far in the subsequent response of the mice to virus. To determine the variability of dosage of an inhaled organism, it seemed of interest to perform a few experiments in which a readily

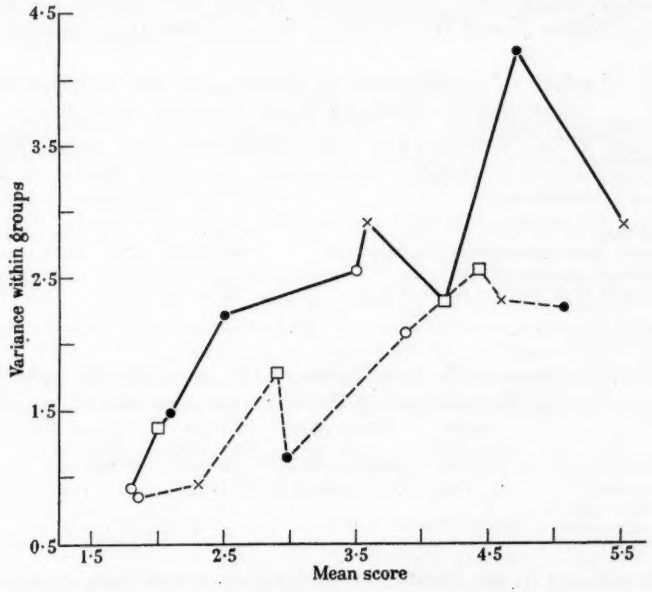

FIGURE.—Variability between the pulmonary scores in mice in relation to the mean pulmonary score.

——— intranasal instillation.
- - - - - - inhalation.
● Experiment 1.
× „ 2.
○ „ 3.
□ „ 4.

identifiable bacterium replaced virus and was recovered from the lungs of mice as soon as the period of exposure ended. The experiments were designed also to yield information on the uniformity of distribution of particles within the cabinet and within the respiratory system of the mouse.

Ten mice placed in different recorded positions within the cabinet were exposed for one hour to a mist containing *Chr. prodigiosum*. This consisted of the centrifuged deposit of 50–100 ml. of a 24- or 48-hr. broth culture of the organism at room temperature re-suspended in 4 ml. saline. At the end of the hour the mice

were killed rapidly and various parts of the lungs removed and weighed separately. These were ground with sand, and saline added to make a 2 per cent suspension. After centrifugation for 10 min. at 1000 G., 0·1 ml. of the supernatant fluid was plated on agar. For as much as possible of the time between death and culture the tissues and suspensions were kept at + 2°.

Three such experiments revealed rather wide variations in the total number of organisms recovered from each mouse, and in the numbers recovered from different parts of the lungs ; differences were visibly less extreme than when a solution of dye was instilled intranasally.

The variations were such that it seemed appropriate to apply the sinh $^{-1}\sqrt{x}$ transformation (Anscombe, 1950), which yielded on analysis the information presented in Tables II and III. From it we concluded that while no consistent

TABLE II.—*Analysis of Variation in the Recovery of* Chr. prodigiosum *from the Lungs of Mice Exposed to Bacteria-containing Mists.*

Source of variation.	Degrees of freedom.	Mean square.
Positions in the cabinet	9	0·461
Experiments	2	1·323
Positions × experiments	18	0·552
Parts of lung	4	0·461
Positions × parts of lung	36	0·099
Experiments × parts of lung	8 (116)	0·126 (0·094)
Experiments × positions × parts of lung	72	0·088
Total	149	

TABLE III.—*Variation in the Distribution of* Chr. prodigiosum *within the Lungs of Mice Exposed to Bacteria-containing Mists.*

	Whole upper right lobe.	Whole middle right and median lobes.	Whole lower right lobe.	Upper half (approx.) left lung.	Lower half (approx.) left lung.
Mean on sinh scale	1·99	1·83	1·72	1·96	1·74
Bacterial count after inverse transformation	13	9·2	7·3	12	7·7

difference occurred in the number of bacteria recovered from mice according to their position in the inhalation cabinet, a significant variation existed between the numbers isolated from different parts of the lung. More bacteria were isolated from the upper parts of the right and left lungs and fewer from the lower parts, with the middle and median lobes (combined) occupying an intermediate position.

If, as seems likely, the variation in the total number of bacteria found in the lung under these conditions may be taken as an estimate of that in the intake of inhaled influenza virus, it is of interest to compare it with the variation obtaining in the pulmonary scores of mice 7 days after inhalation of virus. The various quantities have, of course, to be expressed in comparable terms ; this was achieved after suitable transformation of the pulmonary scores, and using the relation noted previously between the dilution of virus and the resulting mean degree of infection. Expressing both variations in terms of the log dose or log intake of virus, we obtained the following estimates :

(i) a standard deviation of 0·28 for the initial intake of virus, and

(ii) a standard deviation of 0·63 which would be required for the intake of virus in order to account for variability of pulmonary scores on the 7th day, if initial intake were the only source of variability.

It follows that by far the greater part of what we have referred to above as the persisting variability is due to differences in the response of individual mice to infection with influenza. The stock of mice used is heterogeneous, and it is probable that this persisting variability would be reduced by use of a suitable inbred strain.

Other experiments in which the tips and edges of the pulmonary lobes were compared with tissues around and including the hilum afforded evidence that the bacteria are present in the parts most remote from the main bronchi as well as nearer the main bronchi.

Comparison of Nebulizers.

We carried out the preceding work with one nebulizer throughout. Since, however, breakages must inevitably occur and these articles of commerce are visibly not standard, it seemed desirable to compare a number. These were coloured to facilitate identification.

The nebulizers were first measured for the time of emptying at a pressure of 50 cm.Hg. They were then used in different sequence on each of 3 days to infect 72 mice per nebulizer distributed in 24 recorded positions in the cabinet. From the data in Table IV it is apparent that the more slowly emptying yellow nebulizer gave appreciably lesser degrees of infection than did the others, as might be expected if the level of infection is related to the average content of virus in the atmosphere. When, as was done in the Figure, variances were plotted against mean pulmonary scores, there was little difference in variability between atomizers.

TABLE IV.—*Comparison of Different Nebulizers Used in Infecting Mice with Influenza Virus.*

Experiment.	Dilution of virus.	Nebulizer used.		
		Yellow, 44 min.	Red, 25 min.	Blue, 34 min.
1	10^{-2}	2·75 (1·65)	3·75 (2·05)	3·74 (2·20)
2	10^{-2}	2·15 (0·89)	3·15 (1·71)	2·69 (1·51)
3	$10^{-2\cdot3}$	1·74 (0·59)	1·85 (0·72)	2·01 (0·89)

The times following the colours of the nebulizers were those necessary to dispense 4 ml. viral suspension at a pressure of 50 cm. Hg : in all cases exposure of the mice within the cabinet lasted one hour. The figures given are the mean pulmonary scores in the different groups of mice, with the variances in parentheses.

It also appeared that the data in Table IV were such that a log transformation could be applied, when the complication of unequal variances was removed and all means could be compared directly. This was done and the means and variances recalculated, with the results seen in Table V. There are clearly no appreciable differences between nebulizers in the variability of disease produced in the mice. The mean variance of ·0466 gives a standard error of ·216, and as each mean in Table V is an average of 216 results, the 95 per cent limits of error are $\pm$ ·030. There are thus no detectable differences between the red and blue nebulizers, but both give significantly higher pulmonary scores than does the yellow. In

TABLE V.—*Comparison of Different Nebulizers Used in Infecting Mice with Influenza Virus.*

Experiment.	Order of use of nebulizer. 1	2	3	Mean.
1 .	·528 (blue)	·531 (red)	·383 (yellow) .	·481
2 .	·477 (red)	·290 (yellow)	·375 (blue) .	·371
3 .	·202 (yellow)	·257 (blue)	·221 (red) .	·227
Mean .	·392	·359	·326	

Mean yellow = ·292 Mean red = ·400 Mean blue = ·387
Variance yellow = ·0453 Variance red = ·0470 Variance blue = ·0475

The data forming the basis of Table IV are here subjected to log transformation and the means and variances recalculated.

practice the lower level of infection obtained with the slowly emptying nebulizer would be compensated for by using a stronger viral suspension.

These experiments were also planned in such a way as to detect differences in the level of infection according to the position occupied by the mice in the cabinet. Once again no such differences were noted.

SUMMARY.

After intranasal instillation of dye the widest variation exists in the amounts reaching the lungs of different mice; in some animals none can be seen; from others as much as 15 per cent of the inoculum can be recovered.

We describe a simple apparatus capable of consistently and reproducibly infecting large numbers of mice by inhalation of influenza virus. On the seventh day after infection mouse-to-mouse variations in the pulmonary lesions so induced are materially smaller than those following intranasal instillation of virus by experienced workers. The greater part of the variation still persisting is probably due to differences in the response of individual mice to infection.

Tests with *Chr. prodigiosum* in this apparatus showed that more bacteria reach the upper than the lower parts of the lungs, but that the parts most remote from the main bronchi are infected in all lobes. Irregularities of distribution are less than after intranasal instillation of dye.

The use of different nebulizers of the same general pattern does not affect the mouse-to-mouse variation. A slowly emptying nebulizer, however, was responsible for a lower level of pulmonary infection; in practice, this would be compensated for by the use of a stronger viral suspension.

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A STUDY OF CHICK EMBRYO LESIONS PRODUCED BY INFLUENZA VIRUSES.

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DURING an examination of four strains of influenza virus A-prime isolated in London during the 1950–51 epidemic of influenza, it was found that one of the strains, HAL, would regularly kill 10-day chick embryos with pronounced lesions within 48 hours of inoculation by the amniotic route (Smith, Westwood and Belyavin, 1951). This lethal property was very rapidly acquired and was in evidence from the third amniotic pass onwards. Such behaviour was of particular interest, since it was in direct contradiction to the findings of previous workers, who have stated categorically that influenza virus passed amniotically has never produced demonstrable lesions except in the lungs, and that the external appearances of the embryos have invariably been normal (Burnet, 1940*a*, *b* ; Burnet and Foley, 1941 ; van Rooyen and Rhodes, 1948). Lesions of the type described have been reported only from two strains of exalted virulence following prolonged passage on the chorio-allantoic membrane (Burnet, 1936 ; Burnet and Lush, 1938).

Further work showed that the classical PR8 strain would also produce similar lesions when inoculated by this route, and that similar lethal properties were more gradually acquired by another of the new strains, M.W., on continued amniotic passage. It was thought possible that the discrepancy might be due to our use of 10-day embryos as opposed to the 13- or 14-day embryos used by previous workers for amniotic isolation and passage, and that any well-adapted egg-strain of influenza virus might be capable of killing embryos with the production of lesions if embryos of suitable age were used.

The present paper reports experiments designed to put the hypothesis to test and to determine whether the lesions represent a specific effect of influenza virus infection. A fuller description of the lesions is also included.

MATERIALS AND METHODS.

Influenza virus strains.

The virus strains used in this work were Influenza A, PR8 ; Influenza A-prime, F.M.1 and HAL ; Influenza B, Lee. The PR8 and Lee strains have been maintained by allantoic passage in this laboratory since 1947. The F.M.1 strain was received in 1951 by the courtesy of Dr. C. H. Andrewes as an allantoically adapted strain, and has received 5 consecutive passages in this laboratory. Strain HAL was isolated by the amniotic route on January 14, 1951, from washings obtained from a house physician in University College Hospital. Allantoic adaptation was initiated from the 4th amniotic pass and the strain has since been

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maintained by passage by both routes. The amniotic strain has received 30 and the allantoic strain 28 consecutive passages.

Vaccinia virus.

The Lister lymph strain of vaccinia was inoculated on to the chorio-allantoic membrane of 10-day embryos and has since been maintained by membrane passage.

Herpes virus.

An egg-adapted strain of herpes simplex was obtained by the courtesy of Dr. J. A. Dudgeon and has been maintained by membrane passage.

Newcastle disease virus.

The virus was obtained as freeze-dried material from Dr. C. H. Andrewes in 1947. It has been maintained by serial allantoic passage.

Bacterial strains.

The S19 strain of *Brucella abortus* was kindly supplied by Professor J. C. Cruickshank of the London School of Hygiene and Tropical Medicine. The *Staphylococcus aureus* and *Clostridium welchii* strains were from stock cultures kept in this laboratory.

Technique of amniotic inoculation.

Eggs were incubated in an upright position and inoculated through the air sac by the method of Taylor and Chialvo (1942). The shell covering the air-sac was removed and the underlying membrane cleared with sterile liquid paraffin. Fine curved forceps were used to pierce the shell and chorio-allantoic membranes and draw up a tent of amnion into which 0·2 ml. of inoculum was injected by means of a fine-drawn Pasteur pipette. It was found that this method of inoculation was both less laborious and less traumatic than the side approach through an artificial air-sac, and non-specific deaths were fewer.

Penicillin and streptomycin were added to all virus inocula to give a final concentration of 2000 units of each per ml.; thus 400 units of each were injected into each embryo.

Diluents.

Nutrient broth, 10 per cent horse-serum saline or normal saline were used as virus diluents.

Histological technique.

Embryos were fixed whole in alcoholic Bouin's solution immediately after removal from the egg. After dehydration and clearing, heads and bodies were blocked separately in paraffin wax and sections cut at about 4μ. Bodies were sectioned in a sagittal plane chosen to include lung, liver, mesonephros and metanephros in a single section. Heads were cut transversely to show cerebral hemispheres and olfactory lobes. Sections were stained with Mayer's haemalum and eosin.

EXPERIMENTAL.

Lesions Produced in 10-day Chick Embryos.

In the course of experiments with the HAL strain over 200 embryos were examined with regard to their gross lesions, and of these 12 were also subjected to detailed histological study. Histological examinations were also carried out on uninoculated control embryos and embryos inoculated with sterile broth. A further series of control embryos was removed and fixed at 24-hr. intervals from the 8th to the 13th day of incubation.

The virus diluents used, namely normal saline, 10 per cent horse-serum saline and nutrient broth, were found to be without influence on the incidence and severity of the lesions. None of these materials in themselves produced lesions except in a single case in which one of six embryos inoculated with sterile broth showed quite severe lesions the cause of which was not determined.

Macroscopic appearances.

The macroscopic lesions produced by the HAL strain were consistent in character though they varied in severity. The embryos were smaller and more poorly developed than uninoculated embryos of the same age. Their heads and bodies were deeply congested, being purplish or bright scarlet in colour with dilated and prominent blood-vessels. The feather follicles were frequently seen as bright red pin-points, especially over the thorax and along the posterior borders of the wings. Small haemorrhages were often present in the skin and pectoral muscles. The body was occasionally oedematous. On the posterior aspect of the head the venous plexus overlying the brain was nearly always conspicuous as a deep purple mass of grossly engorged vessels clearly visible through the skin.

Dissection showed that the congestion was shared by most organs of the body, excluding the gut. The liver and kidneys, which are normally straw-coloured, were deep purple, with the surface of the latter covered by widely dilated vessels. The lungs were less commonly affected, being frequently normal in appearance but sometimes sharing in the general congestion. In the head the meninges were usually congested and pink ; the brain itself was occasionally pink but more usually appeared normal. Haemorrhage into the brain was not seen.

Microscopic appearances.

In none of the embryos examined was an inflammatory cellular reaction seen in any of the tissues. The striking congestion seen macroscopically was also the most conspicuous histological feature.

Skin.—The skin showed gross congestion of superficial vessels with occasional small haemorrhages. The surface epithelium was degenerated and frequently completely desquamated.

Muscle.—The muscular tissue was congested, with frequent small haemorrhages into the pectoral muscles.

Liver.—Congestion in the liver was usually very conspicuous. In the normal liver the central vessels of the lobules are usually filled with blood, but the sinusoids are empty or contain little blood. The liver cells are cuboidal and the lobular architecture clear. In the pathological livers the main vessels, central veins and sinusoids were all grossly engorged and packed with blood cells. The lobular architecture was disrupted, the liver cells being forced apart and frequently rounded. Many cells were obviously degenerate, with nuclei showing fragmentation, chromatolysis or pyknosis. At the same time many mitotic figures were usually to be seen (Fig. 1 and 2).

Kidney.—The appearance of the kidney was even more striking. In embryos of this age both mesonephros and metanephros are present, the latter being in a stage of active differentiation with developing tubules and glomeruli supported in a connective-tissue stroma which contains well-defined blood vessels. The mesonephros on the other hand is fully differentiated. Large glomeruli and tubules, devoid of connective-tissue stroma, are in direct contact with and completely enveloped in interconnecting blood sinuses derived from the cardinal vein. It was the nature of these sinuses which gave such a dramatic appearance to the pathological kidneys. Their engorgement led in severe instances to wide

separation of individual tubules and glomeruli, which then appeared scattered thinly through a lake of blood. In embryos with severe lesions there was also marked degeneration of tubular epithelium, the cells being separated from each other and the basement membrane and showing degenerative changes in cytoplasm and nuclei. Some tubules were completely stripped of epithelium, while cellular débris was present in all tubules and some contained blood. Even in kidneys showing the most severe damage mitotic figures were common (Fig. 3 and 4).

Owing to its more complex structure the metanephros presented a less dramatic picture but congestion and cellular degeneration were nevertheless obvious.

Lungs.—The lungs were usually relatively normal in appearance, though some degeneration of the para-bronchial epithelium was frequently present. Some degree of congestion was more frequent and in two embryos there were haemorrhages into the lung stroma (Fig. 5 and 6).

Spinal ganglia.—The normal ganglion cell is a large angular cell with a deeply and unevenly staining cytoplasm and a round vesicular nucleus. The nuclear chromatin is arranged in a cartwheel pattern, with a central mass connected by fine strands to a thin peripheral layer of chromatin on the limiting membrane. In pathological embryos the cells were swollen and rounded, with granular degeneration of the cytoplasm, which was pale-staining and sometimes showed tigrolysis. The wheel pattern and vesiculation of the nuclei were lost and replaced by a diffuse granularity, the chromatin masses being blurred, rounded and woolly in outline (Fig. 7 and 8).

These changes were of particular interest, since they seemed to be the earliest to appear in the embryo. Even embryos with minimal changes in other organs sometimes showed detectable nuclear changes in the ganglion cells.

Brain.—In spite of its relatively normal macroscopic appearance, histological changes could usually be detected in the brain. In the normal brain the blood-vessels are collapsed and difficult to pick out, so that even slight congestion in the pathological tissue is conspicuous. The congestion found was patchy and asymmetrical, and might be either in cerebral hemispheres or olfactory lobes. When congestion was present the perivascular spaces were usually dilated. Some degree of nerve-cell degeneration was usually to be seen. No haemorrhages were seen.

Stage of differentiation of embryos.

Although embryos dead from virus infection appeared smaller and more poorly developed than uninfected embryos of corresponding age, it was clear from histological examination that differentiation of the internal organs was not in fact delayed. Comparison of the kidneys, livers and lungs of pathological embryos with those of normal embryos of different ages showed this clearly. The tubules of the mesonephros in pathological embryos were, however, noticeably smaller than those of uninfected embryos. It would seem therefore that infection retarded growth rather than differentiation.

Onset of lesions and relation to post-mortem changes.

In selecting embryos for histological examination it was obviously desirable to take live embryos showing severe lesions, but this proved extremely difficult

The onset of lesions appeared to be a terminal event, and, if embryos were alive at the time of harvesting, they usually showed no lesions or only minimal congestion. Of the embryos examined histologically, nine had died recently for they still possessed good muscular tone. These showed lesions varying from minimal congestion to severe congestion with haemorrhages. The lesions, however, cannot be due merely to post-mortem change, for three embryos were obtained which had severe lesions but whose hearts were beating sluggishly at the time of harvesting. The histological appearances of these embryos resembled closely those of the dead embryos, so that the cellular degeneration described must represent an ante mortem phenomenon. The presence of numerous mitotic figures even in severely damaged tissues, and the fact that in dead embryos showing only minimal congestion there was little or no evidence of cellular degeneration, also support this contention.

Specificity of Lesions.

During the original investigation of the HAL strain it was found that the lesions could be completely prevented by homologous immune serum but not by normal or by heterologous immune serum. On the other hand, apparently identical lesions were observed in embryos dead from PR8 and Newcastle disease virus infection. Moreover, one control embryo which had received only sterile broth was observed with similar lesions (Smith *et al.*, 1951). It is evident therefore that such lesions are not specific to the influenza group of viruses, but represent a non-specific response of the embryo to severe infections or toxaemias of different types.

The following viruses and bacteria have now been tested, and with the exception of *Clostridium welchii* have been found to kill 9- or 10-day embryos with production of lesions macroscopically similar to those already described for the HAL strain.

Viruses.	*Strain.*
Influenza A	PR8.
Influenza A Prime	F.M.1.
	HAL (amniotic).
	HAL (allantoic).
	M.W.
Influenza B	Lee.
Newcastle disease	
Vaccinia	Lister lymph.
Herpes	H.S.C.

Bacteria.

Staphylococcus aureus.
*Brucella abortus S.*19.
Clostridium welchii.

Cl. welchii caused colliquative necrosis of most of the contents of the egg and the embryo was largely disintegrated. The remnants, however, showed intense congestion.

No attempt was made to compare the virulence of the various organisms in terms of infective doses since limitations in the materials available precluded the necessary titrations. All the virus preparations were inoculated at 10^{-2} dilution, representing about 10^6 infective doses of the influenza group of viruses and 10^3 infective doses of vaccinia and herpes. Doses of bacteria were adjusted empirically so as to cause death of the embryo not earlier than 24 hr. Some variation was observed in the intensity of the lesions, particularly in the degree of congestion of the liver, but similar variation has been observed with the HAL strain and appears to be largely attributable to variation in the susceptibility of different batches of embryos.

Effect of Age of the Embryo.

During routine passage of the HAL amniotic strain it was observed that embryos of up to 11 days of age were killed with fair regularity, though the lesions tended to fall off in severity with increasing age of the embryo. When 12-day embryos were used for passage fewer deaths occurred and the lesions were much less striking. These findings indicate that a change in the susceptibility of the chick embryo occurs at about the 12th day of incubation. An experiment was designed to test this point directly.

Four groups of embryos from a single batch were placed in the incubator at 24-hr. intervals and incubation was continued until the first group was 13 days of age, the younger groups being respectively 12, 11 and 10 days of age. All the groups were then inoculated amniotically with 0·2 ml. of 10^{-2} dilution of the F.M.1 strain and observed for 4 days. The experiment was repeated with a second batch of eggs ranging in age from 9 to 13 days and the combined results of the two experiments are shown in Table I. A third experiment using the HAL strain gave essentially similar results.

TABLE I.—*Effect of Age of the Embryo at Time of Inoculation with F.M.1 Strain of Virus.*

Age of embryos in days at time of inoculation.	Number of embryos in group.	Number of embryos dead by—			Number surviving at 96 hr.
		48 hr.	72 hr.	96 hr.	
9	4	0	4	—	0
10	11	5	10	11	0
11	12	1	5	9	3
12	11	1	1	3	8
13	12	3	3	3	9

It is clear that there is a decline in susceptibility of the embryo after 11 days of incubation, but that deaths may still occur even among 13-day embryos. Examination of the embryos themselves revealed moreover that all the embryos, whether alive or dead, showed pathological changes. In the live embryos these changes were confined to the liver, and were the same in all embryos irrespective of age. The normal liver at 12 days is of a pale buff colour, but as the embryo ages it acquires a green tinge until by the 16th day it is a uniform pale green. By contrast, the livers of the live infected embryos were a uniform dark olive-green colour. In two embryos only had the changes progressed beyond this stage. One showed early congestion superimposed on the basic dark green,

while in the other the deep purple of advanced congestion had completely masked the green colour.

Among the dead embryos age exerted a marked influence on the severity of the external lesions and also determined the nature of the macroscopic liver changes. The severity of the external lesions decreased with increasing age of the embryo, the determinant factor being the age of the embryo at death rather than at the time of inoculation. The older embryos showed external congestion only around the feet and beak, though reflexion of the skin over the thorax frequently revealed congestion of the pectoral muscles along the line of their attachment to the keel of the sternum. The liver changes depended both on the age of the embryo at the time of inoculation and the period for which it had survived thereafter. In 10-day embryos surviving less than 48 hr. patchy congestion shaded into the normal buff colour, but in those surviving until the third or fourth day circumscribed areas of deep congestion stood out sharply against a pale cream background. In the older embryos the congestion was progressively less patchy though not reduced in intensity. The older the embryo at the time of inoculation and the longer it had survived before death the more uniform was the congestion.

The significance of these changes has not been determined. Deposition of bile pigment has been observed histologically in both normal and pathological livers, and the deep green of the livers of live infected embryos is presumably due to an exaggeration of a normal physiological process. This is the only change which has been observed in live vigorous embryos infected with influenza virus, but it has not been ascertained whether it also occurs in other infections and represents part of the non-specific reaction of the embryo.

Effect of Age of the Embryo on Newcastle Disease Infection.

In view of the marked change in susceptibility to the HAL and F.M.1 strains with increasing age of the embryo, an experiment was performed to determine whether a similar change occurred in susceptibility to Newcastle disease virus. Batches of embryos at 10, 12 and 14 days of incubation were inoculated with 10^{-2} dilutions of HAL and N. D. V. viruses. The results of the experiment are shown in Table II.

TABLE II.—*Comparison of the Effect of Age of the Embryo on HAL and N. D. V. Infection.*

Virus.	Age of embryos in days at time of inoculation.	Number of embryos in group.	Number of embryos dead by :				Number surviving at 96 hr.
			24 hr.	48 hr.	72 hr.	96 hr.	
HAL	10	8	0	3	5	7	1
	12	7	0	0	0	0	7
	14	7	0	0	0	0	7
N. D. V	10	8	0	8	—	—	0
	12	8	0	8	—	—	0
	14	8	0	2	8	—	0

The 10-day embryos killed by N. D. V. by 48 hr. had obviously been dead for some time, while the 12-day embryos had died very recently. This fact, together with the delay in the deaths of 6/8 of the 14-day embryos, shows that the effect of age of the embryo is not entirely eliminated in Newcastle disease infection,

but there is a clear contrast with the behaviour of HAL. This contrast is also shown in the nature of the lesions produced. In the 10-day embryos the lesions produced by HAL were more severe than those produced by N. D. V. Taken together with the slower killing time of HAL this suggests that HAL is as damaging to the embryo as is N. D. V., but takes longer to reach a lethal concentration, possibly due to a lower growth rate. On the other hand, the lesions in the 14-day embryos killed by N. D. V. were extremely severe, with very deep congestion of the whole embryo. A direct comparison was not possible since all of the 14-day embryos infected with HAL survived, but the 13-day embryos killed by F.M.1 and HAL in previous experiments showed by comparison only insignificant lesions.

DISCUSSION.

Taylor has recently published an investigation into the behaviour in eggs of a number of viruses isolated in Africa and South America by intercerebral inoculation of white mice (Taylor, 1952). Although a detailed description of the lesions produced is yet to be published, all those viruses which killed the embryo did so with generalised congestion and scattered haemorrhages. Taylor also described haemorrhages into the brain even with those viruses which failed to kill the embryo and which produced no generalised lesions. We have never observed brain haemorrhages even in embryos dead from Newcastle disease infection, but it is not possible without further information to say whether this discrepancy is due to the neurotropic character of the viruses which Taylor was studying or to the response of the particular embryos which he was using. It seems clear, however, that a wide variety of infective agents is capable of causing death of the embryo with a lesion complex which is non-specific, being characteristic of the embryo batch rather than of the individual infection.

In the present study histological examination was limited to embryos infected at 10 days of age with the HAL strain of influenza virus. It is quite probable that if similar studies were carried out on other infections some would be found to show specific lesions in addition to the non-specific lesion complex, whilst others might be characterised by specific lesions alone. For instance, Peers, Ransom and Huebner (1952) described chick embryo lesions produced by a group A Coxsackie virus which contrasts sharply with our findings. The Coxsackie strain studied was not lethal to the embryo, but caused massive necrosis of muscle and necrosis of skin and feather buds without apparently any congestion or haemorrhages. These workers also found that the severity of the lesions decreased with increasing age of the embryo, the major change in susceptibility occurring at 10–12 days of incubation.

Decrease in susceptibility of a host with increasing age is common in experimental virus infections. The age change described here involves something more than susceptibility to infection, for large enough doses were used throughout to ensure infection of all the embryos. The change involves rather susceptibility to the lethal effects of the viruses after infection has been established. Moreover, the lethal effects are to some extent divorced from the severity of lesions in the dead embryos. In the experiment comparing HAL with N. D. V. HAL killed only 5/8 of the 10-day embryos in 72 hours while N. D. V. killed 8/8 in 48 hours ; yet the lesions produced by HAL were more severe than those produced by N. D. V. In the older embryos, on the other hand, the lesions produced by

N. D. V. were very much more severe than those caused by HAL or F.M.1. The developing embryo therefore retains full susceptibility to N. D. V., which not only kills but produces severe lesions irrespective of age. During the same period resistance develops to the lethal properties of the influenza viruses, and tissue reactivity is so altered that severe lesions are not produced even in those embryos which do succumb. These findings suggest a metabolic change in the embryo, and there are two obvious ways in which such a change could operate. A comparison of the lesions produced by HAL and N. D. V. in 10-day embryos suggests that at this stage the influenza strains are as damaging to the embryo as N. D. V. Ageing of the embryo might render the tissues less susceptible to the damaging effects of the former virus, while retaining full susceptibility to the latter; alternatively, ageing of the embryo might render the tissues less suitable for the free multiplication of the influenza viruses so that a lethal concentration is seldom attained. The first mechanism would imply a difference in the mode of damaging action, the second a difference in the growth requirements of the two groups of viruses. Since the adult fowl will not support growth of influenza viruses, it is probable that a difference in growth requirements does in fact play a part in the phenomenon. It should be possible to resolve the question by quantitative studies, and establishment of the relationships between growth rate, concentration of virus and lethal effects should prove informative both as regards the metabolic changes in the embryo causing change in susceptibility, and also as regards essential differences between the viruses themselves.

SUMMARY.

A detailed description is given of the lesions produced by the HAL strain of influenza A prime virus in 10-day chick embryos.

Evidence is presented that these lesions are non-specific and may be caused by a wide variety of infective agents.

At 11–12 days of incubation there is a sharp decline in the susceptibility of the embryo to the lethal effects of the influenza viruses. The severity of lesions also falls off as the age of the embryo increases.

Newcastle disease infection is much less influenced by increasing age of the embryo and there is no decrease in the severity of the lesions produced.

EXPLANATION OF PLATES.

FIG. 1.—Normal liver from a control chick embryo killed on the 12th day of incubation. × 100.

FIG. 2.—Pathological liver from an embryo found dead on the 12th day of incubation, 42 hr. after inoculation with the HAL strain of virus. The sinusoids are packed with blood and the normal architecture disorganized. × 100.

FIG. 3.—Normal kidney (mesonephros) from the same embryo as Fig. 1. × 100.

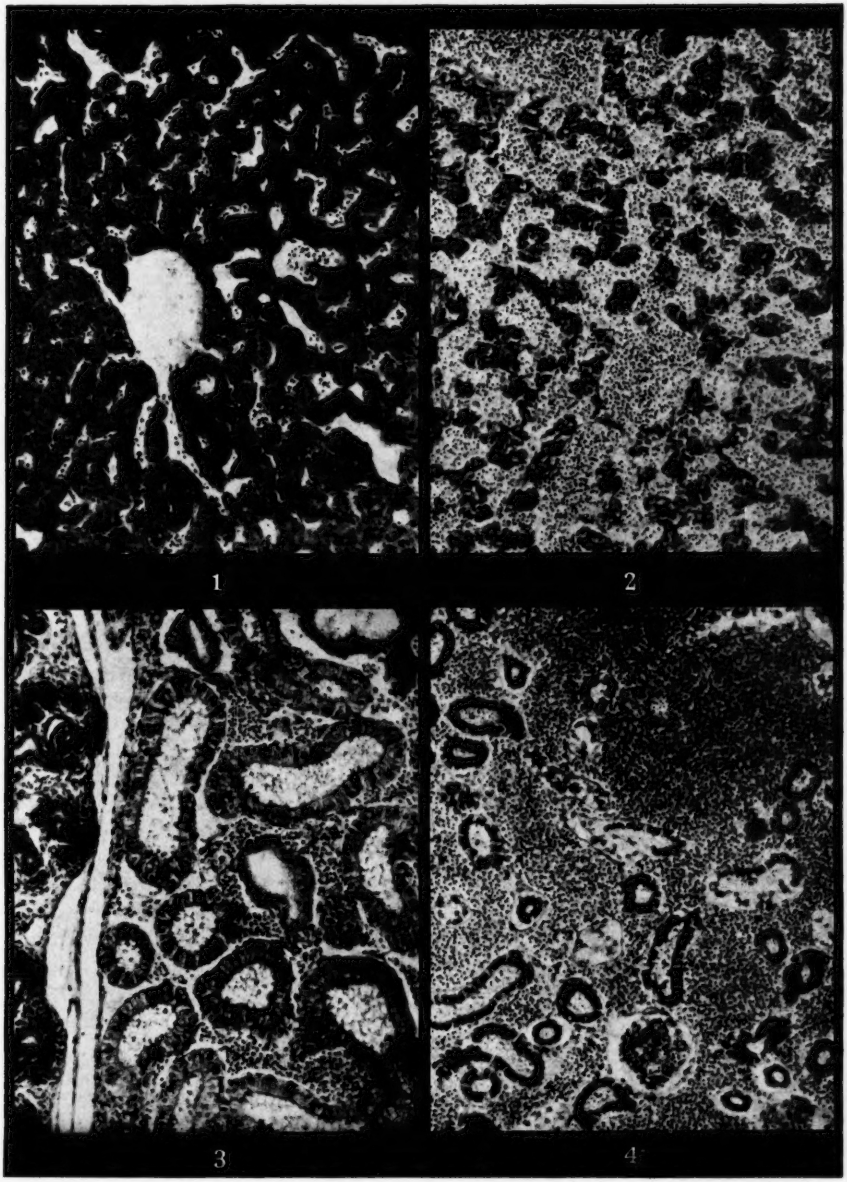
FIG. 4.—Pathological kidney (mesonephros) from the same embryo as Fig. 2, showing gross congestion and tubular degeneration. × 100.

FIG. 5.—Normal lung from the same embryo as Fig. 1. × 100.

FIG. 6.—Pathological lung from one of two embryos which showed severe congestion, with haemorrhages into the lung stroma, following infection with the HAL strain of virus. The embryo was inoculated on the 10th day of incubation and died within 42 hr. × 100.

FIG. 7.—Normal ganglion cells from spinal root ganglion of a control embryo killed at the 12th day of incubation. × 1100.

FIG. 8.—Pathological ganglion cells, showing marked degenerative changes following infection with the HAL strain of virus. The embryo was inoculated on the 10th day of incubation and died within 42 hr. × 1100.



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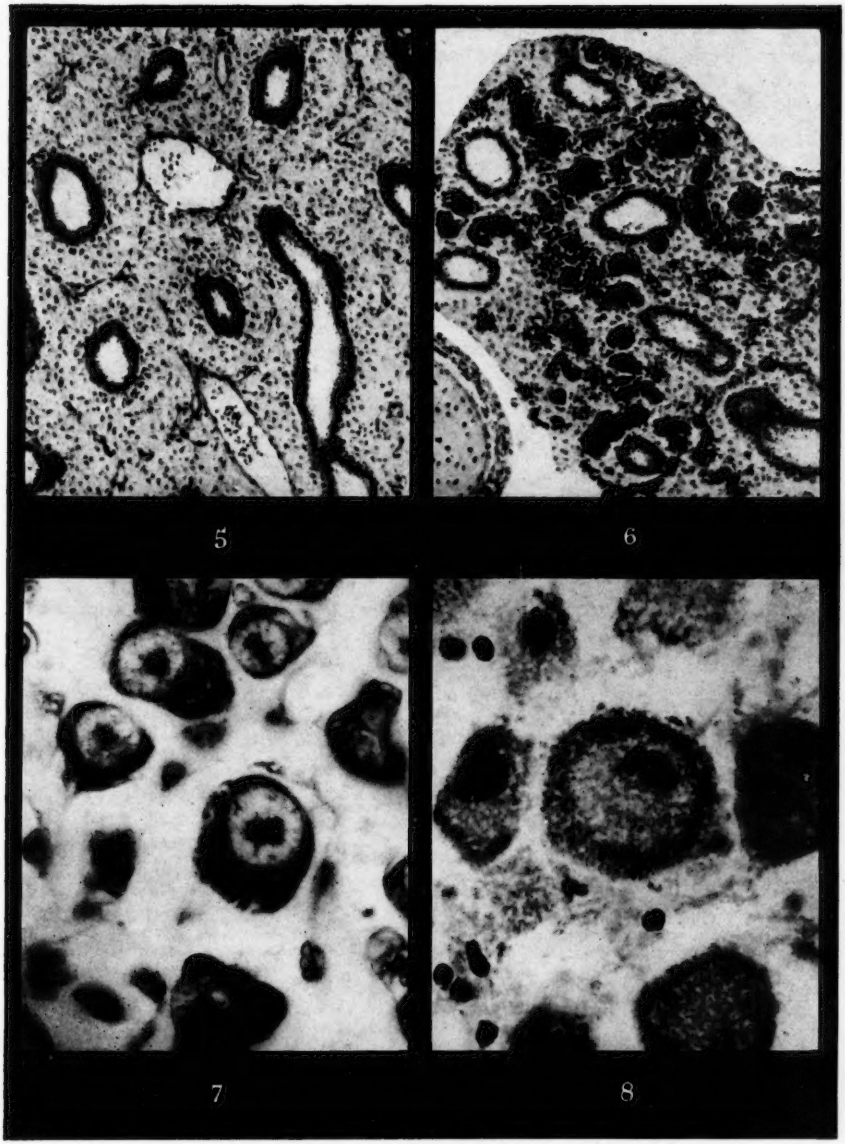

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The implications of the differences in behaviour between influenza virus and N. D. V. are discussed.

I am indebted to Professor Wilson Smith for his advice and criticism, and to Dr. G. Belyavin for help in the study of the histological material.

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